IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Nicholas M. Anstey *et al.*	**Examiner:**	Jacob Cheu
Serial No.:	09/124,485	**Art Unit:**	1641
Filed:	July 19, 1998	**Docket:**	73-97

For: A METHOD OF PROPHYLAXIS AND TREATMENT

Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Nicholas Mark Anstey, declare as follows:

1. That I am a co-inventor of subject matter disclosed and claimed in the above-identified patent application together with Joseph Brice Weinberg and Donald L. Granger.

2. That I am a Professor and Principal Research Fellow at the Menzies School of Health Research and that I have extensive experience in the area of malarial infection and its treatment. Attached hereto is a copy of my brief *curriculum vitae* and track record document.

3. That, in collaboration with colleagues, I have carried out the clinical studies described below which demonstrate that L-arginine is indeed effective in treating moderately severe falciparum malaria in patients (Supplement to the American Journal of Tropical Medicine and Hygiene, Vol 75; 2006: in press).

In patients with moderately severe or severe malaria, parasitized red blood cells adhere *via* endothelial cell receptors to the lining of microvascular blood vessels and there obstruct blood flow. Cytoadherence is now known to take place with all *Plasmodium* blood stages, but is particularly deleterious in the second half of the 48-hour red blood cell portion of the *Plasmodium* life cycle. Initially, the trophozoite grows and breaks down red blood cell contents. Nuclear division and segmentation produces an increase in the number of parasites. The infected red blood cell eventually ruptures releasing merozoite stages which invade further red blood cells. Repeated cycles result in a massive increase in the number of

parasites. *Plasmodium* parasites induce the production of inflammatory cytokines in the host that cause pathology, including endothelial inflammation and damage. Endogenous endothelial nitric oxide (NO) reduces cytokine-induced endothelial expression of adherence molecules including VCAM-1, E-selectin and ICAM-1 (Decaterina *et al.*, J. Clin. Invest., *96*(1):60-68, 1995 referred to in the specification), all of which mediate cytoadherence of parasitized red blood cells (Berendt *et al.*, Nature, *341*:57-59, 1989 referred to in the specification). NO also inhibits cytokine induced endothelial inflammation (Gibaldi, J. Clin. Pharmacol., *33*:488-496, 1993 attached hereto).

Ho *et al.* (Ho *et al.*, Infection and Immunity, *59*(3):873-878, 1991 attached hereto) working with *Plasmodium falciparum* malaria, described the process of sequestration as the "central pathological event in human falciparum malaria" (line 1). Ho *et al.* showed that the *in vitro* C32 human melanoma cell cytoadherence assay was useful in predicting *in vivo* disease severity (see page 876, column 2, first full paragraph). Parasitized blood from subjects with severe malaria had more cytoadherent red blood cells than subjects with uncomplicated malaria. Blood from subjects with cerebral malaria did not show significantly greater levels of binding to C32 cells, but this was predicted and was subsequently confirmed to be because C32 melanoma cells do not express significant ICAM-1 which is the major endothelial receptor used by infected red cells in the brain. Others, for example Udomsangpetch *et al.*, (Journal of Infectious Diseases, *173*:691-698, 1996 attached hereto) used *in vitro* cytoadherence assays to evaluate the ability of anti-malarial drugs to alleviate or prevent pathological adherence processes in malaria. Some drugs which are effective against *Plasmodium* parasites *per se* also reduce cytoadherence *in vitro*, while others do not. At the priority date, I and other scientists working in the field of malaria extrapolated from the experimental results of *in vitro* cytoadherence assays to i) whether a particular drug would affect cytoadherence in human subjects *in vivo* or ii) whether a particular isolate would be likely to be more or less virulent in human subjects *in vivo*, with cytoadherence being correlated with virulence.

The pathophysiological consequences of microvascular obstruction caused by cytoadherence and sequestration depend upon where in the body obstruction occurs. In the brain, convulsions, coma and death may follow obstruction making "cerebral" malaria a dangerous form of malaria. In the kidneys, renal failure may eventuate and in the lungs, pulmonary oedema, both of which can lead to death. Microvascular obstruction prevents adequate blood

flow causing ischemia and hypoxia. Without oxygen, tissues respire anaerobically producing elevated levels of lactate, causing metabolic acidosis and consequent impairment of vital cellular functions (see Krishna *et al*, Transactions of the Royal Society Tropical Medicine and Hygiene, *88*:67-73, 1994, page 71, column 1, third full paragraph referred to in the specification). Endothelial cells lining the microvascular blood vessels respond to signals of ischemia and hypoxia by stimulating dilation of the blood vessel. This effect is mediated by NO in the microvessel environment (see for example, Moncada *et al.*, New Eng. J. Med., *329*:2002-2012, 1993 referred to in the specification). Severe malaria is associated with endothelial inflammation which is exacerbated by sequestration of parasites and microvascular obstruction. In treating subjects with severe malaria, it may be important to rapidly reduce the pathology associated with cytoadherence as well as just kill parasites *per se*.

Prior to the author's publication of Anstey *et al.*, Journal of Experimental Medicine, *184*:557-567, 1996 (hereinafter "Anstey" referred to in the specification) there were no advocates for treating severe malaria by administering to infected subjects an agent that increases NO levels in the subject. It was appreciated that parasites were killed *in vivo* by mediators of the immune response, including endogenous NO, but it was widely believed that NO was a major contributor to the symptoms and pathology of severe malaria such as convulsions, coma, acidosis and hypoglycemia (reviewed by Clark IA and Rockett KA, Advances Parasitology, 1996; 37: 1-56, referred to in the specification). Anstey disclosed unexpectedly that low levels of NO are associated with severe malaria and high levels are associated with reduced pathology. The present invention was predicated, in part, upon this observation together with the background knowledge in the art (as set forth above) that NO is i) required to allow blood vessels to dilate in response to factors that reduce blood flow and ii) to inhibit the production of endothelial adhesion molecules on the lining of blood vessels.

The present invention is directed to the treatment of severe or moderately severe malaria by reducing or inhibiting the pathologic adherence properties of parasitized cells, and/or ameliorating the symptoms and pathology consequent to this. By reducing or preventing cytoadherence and hence sequestration of parasites this prevents the consequent pathophysiological processes occurring in malaria, such as ischemia and hypoxia due to blocked microvessels and inflammation. Moreover, by restoring to endothelial cells the capacity to appropriately vasodilate in response to this cytoadherence-induced ischemia, an

additional benefit of this invention would be to enhance blood flow through parasite-obstructed blood vessels, further ameliorating cytoadherence-induced ischemia and pathology. A reduction in cytoadherence probably also increase splenic clearance of parasites. The method comprises administering an agent that enhances the level of NO in a subject sufficient to inhibit or reduce the pathologic adherence properties of parasitized red blood cells, and and/or ameliorating the symptoms and pathology consequent to this. The agent is L-arginine, NO gas and/or an S-nitrosothiol compound.

It must be appreciated that a direct *in vivo* measure of a reduction in cytoadherence cannot be taken in human subjects with moderately severe or severe malaria. The marker of choice for evaluating the efficacy of administered L-arginine in inhibiting cytoadherence would be one that most closely acts as a marker for changes in the pathophysiological processes occurring in malaria as a result of cytoadherence, such as ischemia. In the clinical trials described below we used peripheral arterial tonometry (PAT) that has recently been validated as a novel non-invasive tool to assess endothelial function in cardiovascular disease. We also used further established markers of malaria disease severity, namely lactate and plasma ICAM-1 levels.

Peripheral arterial tonometry provides a non-invasive method of measuring the capacity of blood vessels to dilate in response to obstructed blood flow. The capacity of blood vessels to respond to ischemia depends upon NO levels in the region. If the microvascular blood vessels in subjects are obstructed, the normal physiological response would be to dilate the blood vessels to facilitate greater blood flow. If the subject is NO deficient, this response does not occur and lactate levels and ICAM-1 levels would be expected to increase in the subject. High plasma lactate levels are a measure of impaired tissue perfusion. Elevated ICAM-1 concentrations are a measure of endothelial cell inflammation. Both increased lactate (Krishna *et al*, 1994 (*supra*)) and ICAM-1 (Jakbosen et al, Immunology, 83: 665-9, 1994) levels are accepted markers of malarial severity.

In a recent clinical study of malaria patients at Mitra Masyarakat Hospital in Timika, Papua, Indonesia, we found that PAT analysis provided an accurate measure of malaria severity useful for evaluating the effects of anti-malaria treatments. The ability of subjects to dilate blood vessels after ischemic stress was least in patients with severe malaria (n=49) compared to patients with moderate malaria (n=72) and greatest in healthy controls (n=50). Blood

analysis from subjects with severe malaria also showed that the PAT values were inversely correlated with plasma lactate (r=-0.31; p=0.01) and ICAM-1 concentrations (r=-0.31; p0.001). High plasma lactate levels are a measure of impaired tissue perfusion. Elevated ICAM-1 concentrations are a measure of endothelial cell inflammation. Thus, the more severe the malaria, the lower the PAT value and the higher the lactate and ICAM-1 levels. In this study, subjects were given a standard anti-malarial treatment and, in the severe malaria group, the PAT values returned to a normal level within 48 hours of receiving treatment. This shows that PAT analysis provided an accurate measure of malaria severity useful for evaluating the effects of anti-malaria treatments.

We also measured NO production directly using a nitric oxide (NOIX) analyser which measures exhaled NO in parts *per* billion. In a longitudinal study conducted at Mitra Masyarakat Hospital in Timika, Papua province Indonesia, adult patients (age 18-60 years) with uncomplicated and severe malaria underwent serial bedside measurement of exhaled NO in parts per billion (ppb) using the NIOX apparatus and American Thoracic Society Guidelines. Measurement required the ability to sit and to cooperate with the exhalation technique and was not possible in subjects with cerebral malaria or prostration. Baseline measurements were possible in 60 patients with moderately severe malaria (patients requiring inpatient parenteral therapy but without WHO manifestations of severe malaria) and 12 with modified WHO criteria for severe malaria. Median exhaled NO was lower in severe malaria [10.5 ppb (IQR: 9.5-15.0)] than moderately severe malaria [18.5 ppb (IQR: 11.1-26.9)]; p=0.03. By 48 hours after standard anti-malarial treatment, exhaled NO in patients with severe disease had increased to levels comparable to those found in healthy controls (median 16.6 [IQR: 11.9-27.0]).. Real-time bedside measurement of exhaled NO allows direct measurement of NO production in malaria, and demonstrates impaired production in patients with severe malaria compared to those with moderately severe malaria. The results are consistent with a protective role for NO in malaria. Measurement of exhaled NO has potential utility in evaluating interventions targeting increased NO production in severe malaria.

I report the results of a recent clinical trial which was conducted to test *inter alia* the safety and efficacy of L-arginine in the treatment of moderately severe *falciparum* malaria in patients in Mitra Masyarakat Hospital in Timika, Papua, Indonesia. If L-arginine administration is effective in reducing the pathophysiological consequences of malaria, NO

levels should be enhanced and the ability of the blood vessels to respond to ischemia by dilation should also be enhanced.

Thirty adults (18-60 yrs) hospitalized with moderately severe falciparum malaria (but without WHO criteria of severe malaria) at Mitra Masyarakat Hospital in Timika, Papua, Indonesia were enrolled in a single ascending dose response study to assess safety and preliminary efficacy of adjunctive arginine. Endothelial function was measured by peripheral arterial tonometry (PAT) of the digital microvasculature before and after an ischemic stress, generating a reactive hyperemia PAT (RH-PAT) ratio. A ratio of 1.67 has previously defined endothelial dysfunction. L-arginine hydrochloride was given intravenously over 30 mins at doses of 3g (n=10), 6g (n=10) and 12g (n=10). Endothelial function by PAT, exhaled NO and plasma arginine were compared before and after arginine infusion. Results were also compared with those of a control group of patients (n=42). Arginine infusion resulted in a significant 55% increase in exhaled NO (p=0.0001) and a 19% improvement in endothelial function in the group overall, with the mean RH-PAT index increasing from 1.76 (95% CI 1.62-1.89) to 2.06 (95% CI 1.84-2.25) (p=0.01). In a prospectively defined patient subgroup with impaired endothelial function (RH-PAT index <1.67; n=14), endothelial function improved 38% (p=0.004), with the increase in RH-PAT index being dose related. There were no clinically significant changes in vital signs, pH, glucose or K+ after infusion.

Arginine infusion up to 12g is safe and can improve NO production and endothelial function in adult patients with moderately severe malaria.

I conclude from the results of the above-described clinical trials that L-arginine is effective in treating moderately severe malaria. Further, I expect that L-arginine and other agents that enhance the level of NO production in a human subject will be effective in the treatment of severe malaria by inhibiting or reducing pathologic adherence properties in malaria subjects, and/or amelioration of clinical symptoms.

4. That all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code and

that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

13 July 2006
Dated

Nicholas M. Anstey

Brief CV: Professor Nicholas ANSTEY PhD FRACP MSc DTM&H MB BS(Hons)

A. PERSONAL DETAILS:

NAME: **Nicholas M Anstey** | TITLE: Professor

CURRENT POSITION:

Clinical positions:

1. Infectious Diseases Physician and Staff Specialist, Division of Medicine, Royal Darwin Hospital (RDH)
2. Visiting Specialist Physician, Galiwin'ku Community, Elcho Island, East Arnhem Land, NT

Academic positions:

1. Head, International Health Program, Principal Research Fellow, Menzies School of Health Research: 2000-
2. Honorary Professor, Faculty of Health Sciences, University of Queensland: 2004-

B. EDUCATION:

INSTITUTION	DEGREE	YEAR	FIELD OF STUDY
University of Western Australia	MB,BS (Hons)	1985	Medicine
University of London	MSc	1989	Tropical Medicine
Royal College of Physicians	DTM&H	1989	Tropical Medicine
Royal Australasian College of Physicians	FRACP	1992	Infectious Diseases
University of Western Australia	PhD	2000	Malaria pathophysiology

C. POSTGRADUATE PROFESSIONAL EXPERIENCE:

1989-1990: **Clinical Research Officer**, Medical Research Council Laboratories, The Gambia, West Africa
1990-1993: **Infectious Diseases Registrar**, Royal Darwin Hospital, NT and Sir Charles Gairdner Hospital, WA
1993-1994: **Clinical Fellow in Adult Infectious Diseases**, Duke University Medical Center, NC, USA.
1994-1995: **Clinical Research Fellow**, Duke-Muhimbili Clinical Research Laboratory, Dar es Salaam, Tanzania
1995-1996: **Clinical Research Fellow in Infectious Diseases**: Division of Infectious Diseases, Duke University Medical Center, Durham, North Carolina, USA.
1996-present **Head**, International Health Program, Menzies School of Health Research (MSHR).
1996-present **Senior Research Fellow (1996-2000) and Principal Research Fellow (2000-)**, MSHR

Awards:

1994 American Society of Tropical Medicine & Hygiene Fellowship in Tropical Medicine
1999 Australasian Society for Infectious Diseases Award for Advanced Research
2001 University of Western Australia Robert Street Prize for Excellence in Research
2002 NHMRC Practitioner Fellowship

D. GRANT SUPPORT:

Funder	TITLE	Period of Support	Total $ Value
Current Funding:			
NHMRC Program Grant 290208	Tropical Infectious Diseases – pathogenesis and vaccine research	2004-2008	$8,853,837
NHMRC/Wellcome Trust International Collaborative Research 283321 (NHMRC component)	Research and training to reduce morbidity and mortality from malaria in Papua (Indonesia) and Papua New Guinea	2004-2008	$1,649,830
NHMRC/Wellcome Trust International Collaborative Research 283321 (Wellcome comp.)	Research and training to reduce morbidity and mortality from malaria in Papua (Indonesia) and Papua New Guinea	2004-2008	$1,502,089
NHMRC Practitioner Fellowship	Pathophysiology and treatment of malaria and other tropical infectious diseases in our region	2003-2007	$447,125
National Institutes of Health (USA)	Nitric oxide and severe malaria II	2004-2008	US$665,000
Australian Research Council	ARC Network for Parasitology	2005-2009	$2,465,261
Community Health & Anti-Tuberculosis Association (CHATA)	Nutritional intervention to improve tuberculosis treatment outcome in Timika Indonesia	2006	$51,000
Past Funding:			
National Institutes of Health	Nitric oxide and severe malaria I	1997-2003	US$714,755
CHATA	Pulmonary Disability in TB Patients	2003-2004	$85,817

EXHIBIT
tabbies
A

E. SELECTED RECENT PUBLICATIONS (from 40 in past 5 years):

NA1. Tjitra E, Suprianto S, Currie BJ, Morris P, Saunders J, **Anstey NM**. Therapy of uncomplicated *falciparum* malaria: A randomised trial comparing artesunate plus sulfadoxine-pyrimethamine *versus* sulfadoxine-pyrimethamine alone in Papua, Indonesia. *Am J Trop Med Hyg* 2001; 65: 309-317.

NA2. **Anstey NM**, Currie BJ, Hassell M, Palmer D, Dwyer B, Seifert H. Community-acquired bacteremic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii*, with throat carriage in at-risk groups. *J Clin Micro* 2002; 40: 685-686.

NA3. **Anstey NM,** Jacups SP, Cain T, Pearson T, Zeising PJ, Fisher DA, Currie BJ, Marks PJ, Maguire GP. Pulmonary manifestations of uncomplicated falciparum and vivax malaria: cough, small airways obstruction, impaired gas transfer, and increased pulmonary phagocytic activity. *J Infect Dis* 2002; 185: 1326-34.

NA4. **Anstey NM**, Boutlis CS, Saunders JR. Systemic nitric oxide (NO) production in human malaria: 1. Analysis of NO metabolites in biological fluids. *Methods Mol Med* 2002; 72: 469-74.

NA5. Morahan G, Boutlis CS, Huang D, Pain A, Saunders JR, Hobbs MR, Granger DL, Mwaikambo ED, Marsh K, Roberts DJ, **Anstey NM.** A promoter polymorphism in the gene encoding interleukin-12 p40 (*IL12B*) is associated with mortality from cerebral malaria and reduced nitric oxide production. *Genes Immun* 2002: 3: 414-418.

NA6. Tjitra E, Baker J, Suprianto S, Chen Q, **Anstey NM.** Therapeutic efficacy of artesunate plus sulfadoxine-pyrimethamine and chloroquine plus sulfadoxine-pyrimethamine in pilot studies in vivax malaria: relationship with *Plasmodium vivax dhfr* mutations. *Antimicrob Agents Chemo* 2002; 46: 3947-3953.

NA7. Hobbs M, Udhayakumar V, Levesque M, Booth J, Tkachuk A, Pole A, Coon H, Roberts JM, Karuiki S, Nahlen BL, Mwaikambo ED, Lal AL, Granger DL, **Anstey NM,** Weinberg JB. A novel NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children. *Lancet* 2002; 360: 1468-1475.

NA8. Lopansri BK, **Anstey NM**, Weinberg JB, Hobbs MR, Levesque MC, Mwaikambo ED, Granger DL. Low plasma arginine levels in children with cerebral malaria and decreased nitric oxide production. *Lancet* 2003; 361: 676-8.

NA9. Boutlis CS, Fagan PK, Gowda DC, Lagog M, Mgone CS, Bockarie MJ, **Anstey NM**. Immunoglobulin G responses to *Plasmodium falciparum* glycosylphosphatidylinositols are short-lived and predominantly of the IgG_3 subclass. *J Infect Dis* 2003; 187: 862-5.

NA10. Boutlis CS, Tjitra E, Maniboey H, Saunders JR, Suprianto S, Weinberg JB, **Anstey NM**. Nitric oxide production and mononuclear cell nitric oxide synthase activity in malaria-tolerant Papuan adults. *Infect Immun* 2003: 71: 3682-9.

NA11. Davis JS, Currie BJ, Fisher DA, Huffam SE, **Anstey NM**, Price RN, Krause VL, Zweck N, Lawton PD, Snelling PL, Selva-nayagam S. Prevention of opportunistic infections in immunosuppressed patients in the tropical Top End of the Northern Territory. *Commun Dis Intell* 2003; 27: 526-532.

NA12. Cheng AC, Stephens DP, **Anstey NM,** Currie BJ. Adjunctive granulocyte colony stimulating factor for septic shock due to melioidosis. *Clin Infect Dis.* 2004; 38: 32-37.

NA13. Douglas MW, Lum G, Fisher DA, **Anstey NM,** Currie BJ. Epidemiology of community-acquired and nosocomial bloodstream infections in tropical Australia: a 12-month prospective study. *Trop Med Int Health.* 2004; 9: 795-804.

NA14. Cheng AC, Fisher DA, **Anstey NM**, Stephens, DP, Jacups, SP, Currie, BJ. Outcomes of patients with melioidosis treated with meropenem. *Antimicrob Agents Chemother* 2004; 48: 1763-65

NA15. Cheng AC, Jacups SP, **Anstey NM,** Currie BJ. A proposed scoring system for predicting mortality in melioidosis. *Trans R Soc Trop Med Hyg* 2004; 97: 577-581.

NA16. Boutlis CS, Weinberg JB, Baker J, Bockarie MJ, Mgone CS, Cheng Q, **Anstey NM**. Nitric oxide production and nitric oxide synthase activity in malaria-exposed Papua New Guinean children and adults shows longitudinal stability and no association with parasitemia. *Infect Immun* 2004; 72: 6932-6938.

NA17. Elliott JH, **Anstey NM**, Jacups SP, Fisher DA, Currie BJ. Community-acquired pneumonia in northern Australia: low mortality in a tropical region using locally developed treatment guidelines. *Int J Infect Dis* 2005; 9: 15-20.

NA18. Perkins DJ, Hittner JB, Mwaikambo ED, Granger DL, Weinberg JB, **Anstey NM.** Impaired systemic prostagalandin E2 production in children with cerebral malaria. *J Infect Dis* 2005; 191:1548-57

NA19. **The SEAQUAMAT Trial Group.** Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. *Lancet* 2005; 366: 717-725

NA20. Maguire GP, Handojo T, Pain MCF, Kenangalem E, Price RN, Tjitra E, **Anstey NM**. Lung injury in uncomplicated and severe malaria: a longitudinal study in Papua, Indonesia. *J Infect Dis* 2005; 192: 1966-7.

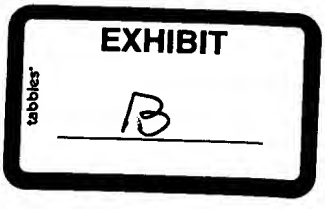
NA21. Lopransri BK, **Anstey NM,** Stoddard GJ, Mwaikambo ED, Boutlis CS, Tjitra E, Maniboey H, Hobbs MR, Levesque MC, Weinberg JB, Granger DL. Elevated plasma phenylalanine in severe malaria: implications for pathophysiology of neurological complications. *Infect Immun;* 2006: 74:3355-9.

Professor Nicholas Anstey: track record

Following my return from the USA and Africa to Darwin in 1996, I have established establish the International Health Programme at Menzies School of Health Research. With the support of the NIH, NHMRC and Wellcome Trust, the emphasis of our research has been translational malaria work with local collaborators in Papua Province, Indonesia. We have established agreements and staff exchanges with all levels of the Indonesian Ministry of Health, building the MSHR-Ministry of Health Research Unit and field laboratory in Timika, Papua (formerly Irian Jaya) which has over 15 resident medical, nursing, laboratory research and support staff. I have completed a large number of field studies and training exchanges mainly with our Indonesian collaborators and students, but have also established links with the PNG Institute of Medical Research, University of PNG and Ministry of Health in East Timor. My collaborative diagnostic and treatment studies with the Indonesian Ministry of Health have already resulted in changes in policy. I was responsible for the inclusion of our Indonesian field site in the multicentre South East Asian Quinine vs Artesunate Severe Malaria Trial (SEAQUAMAT). The results of this trial resulted in a change in WHO's Global 2006 Malaria Treatment Policy. The involvement of our field site meant that Indonesian national drug treatment policy changed before the results of this study were published in *Lancet.* My liaison with National Therapeutic Guidelines has resulted in a similar change in Australian national policy for the treatment of severe malaria (scheduled for publication in August 2006).

My commitment to translational research is demonstrated by the fact that my NIH-funded nitric oxide-malaria pathophysiology studies over the last 12 years have led to NHMRC-Wellcome Trust funded clinical trials of adjunctive arginine treatment for falciparum malaria. The first two stages of these trials have now been completed at our Timika field site in Indonesia. These show that arginine administration is safe in moderately-severe malaria, results in increased nitric oxide production and dose-dependent reversal of endothelial dysfunction. I am Principal Investigator on the 2004-08 NHMRC-Wellcome Trust International Collaborative Research Program Grant with partners in Indonesia.

My work has also been recognised with the award of an NHMRC Practitioner Fellowship in 2003, and research awards from the Australasian Society of Infectious Diseases (1999) and the University of Western Australia (2001). I have been an invited to speak on my malaria research by the Infectious Diseases Society of America, Multilateral Initiative on Malaria, the NIH International Centers for Tropical Disease Research in Washington DC, as well as Australian and Indonesian national and international meetings. Through ongoing collaborations with Indonesian, PNG, Australian and US colleagues I aim to continue to link basic and clinical/field research to address major problems in malaria.



What is Nitric Oxide and Why Are So Many People Studying It?

Milo Gibaldi

From social outcast to citizen of the year in less than a decade is the stuff of fiction. That is precisely what has happened, however, to a remarkably simple molecule, nitric oxide. Nitric oxide is still an environmental pollutant, suspected carcinogen, and precursor of acid rain, but biologists are looking past its dark side. They now see a molecule that is uniting neuroscience, physiology, and immunology. Its ubiquitous distribution in the body and its multifaceted roles are revising our understanding of how cells communicate and protect themselves. This report examines nitric oxide's role in physiology and pathophysiology and reviews novel therapeutic approaches which involve inhibition or induction of the activity of endogenous nitric oxide.

Nitric oxide (NO) is a simple but highly reactive endogenous chemical. In the late 1980s, investigators learned that NO serves as the mediator whereby macrophages express cytotoxic activity against microorganisms and neoplastic cells.[1] Activation of macrophages by cytokines or endotoxin results in the expression of an enzyme, now called NO synthase (NOS). Nitric oxide synthase converts L-arginine to L-citrulline and NO. Inhibition of NO formation by removal of arginine or by addition of N^G-monomethyl-L-arginine (L-NMMA), an NOS inhibitor, blocks the cytotoxic activity of macrophages.[20]

Evidence now is mounting that this unlikely agent, with a biologic half-life of a few seconds, is an important neurotransmitter, perhaps the first to be discovered in a new class of neurotransmitters. Nitric oxide synthase activity has been found in endothelial tissue of the cardiovascular system and in the central nervous system, suggesting an important role for NO in human physiology and pathophysiology.[3]

Nitric Oxide: The Endogenous Nitrovasodilator

We now know that nitric oxide is a mediator of blood vessel relaxation. Acetylcholine, bradykinin, adenosine triphosphate, and other stimuli that dilate blood vessels act on receptors located on the surface of endothelial cells to trigger the release of local hormones that diffuse to adjacent smooth muscle to evoke relaxation. These stimuli lose their vasodilating activity in blood vessels stripped of endothelium. One of the local hormones released in this process is endothelial-derived relaxing factor (EDRF). Endothelial-derived relaxing factor has been identified definitively as either NO or a close derivative that releases NO. Nitric oxide relaxes smooth muscle in blood vessels by activating guanylate cyclase and thereby stimulating the formation of cyclic guanosine 3', 5'-monophosphate (cGMP).[2] Nitric oxide now is considered to be the endogenous nitrovasodilator.[4] In the vasculature, NO also inhibits platelet aggregation and adhesion.[4]

Bassenge has reported that in various ischemic diseases with impaired endothelial function (e.g., hypercholesterolemia, hypertension, reperfusion injury, etc.), either EDRF release is suppressed or the released nitric oxide is immediately inactivated by hemoglobin, as a result of NO's avid affinity for the iron in heme, or by oxygen-derived free radicals before it can affect the vasculature. This sets the stage for reduced vasodilatation and even excessive vasoconstrictor tone, leading to an inadequate blood supply and ischemic damage.[5]

The NO-mediated vasodilator effect of acetylcholine is dramatically reduced in atherosclerotic vessels of nonhuman primates fed an atherogenic diet for several months. Response is restored when the animals are switched to a low-fat diet.[6] A reduced response is seen also in animals with hypertension.[7] This can be prevented by instituting antihypertensive therapy.[8] In the coronary circulation of the dog, occlusion of a major artery for 20 minutes followed

From the School of Pharmacy, University of Washington, Seattle, Washington. Address for reprints: Milo Gibaldi, School of Pharmacy, University of Washington, Seattle, WA 98195.

by reperfusion is associated with a marked decrease in endothelium-dependent relaxation.[9] An impaired NO-mediated response also has been observed in patients with hypercholesterolemia, atherosclerosis, or hypertension.[10]

Exogenous Nitrovasodilators

Nitroglycerin, isosorbide dinitrate, and other nitrate vasodilators are ultimately biotransformed to NO and are widely used in the treatment of coronary artery disease to substitute for a diminished endothelial EDRF/NO production. Nitrates stimulate the formation of cGMP, initiate relaxation, and compensate for insufficient endothelial NO production or for excessive NO inactivation. Luscher observed that the action of nitrate vasodilators is "particularly pronounced in blood vessels with a low basal production of nitric oxide and is enhanced after removal of the endothelium."[11] By the same token, it has been demonstrated in rabbit hearts that inhibition of EDRF synthesis with L-NMMA potentiates the vasodilator response to nitroglycerin.[12]

Continuous administration of organic nitrates leads to tolerance both in the coronary arteries and in the venous system. Nitroglycerin and related nitrates undergo a complex biotransformation requiring sulfhydryl groups to release nitric oxide. This mechanism appears to be susceptible to tachyphylaxis, perhaps because of depletion of the intracellular sulfhydryl pool, and may play an important role in nitrate tolerance. Tolerance results in diminished venodilation and less reduction of preload during nitrate administration. Although the responses to nitroglycerin and related nitrates diminish after the induction of tolerance, the responses to "endogenous nitrate" EDRF formed from L-arginine are not affected by the induction of tolerance.[5,9]

Nitric oxide also mediates the hypotensive effects of nitroprusside. When nitroprusside comes in contact with red blood cells, the molecule decomposes, releasing nitric oxide. Sodium nitroprusside is used primarily to treat hypertensive emergencies, but the drug can be used in many situations when short-term reduction of cardiac preload or afterload is desired.[13]

Organic nitrates do not release NO spontaneously, requiring instead some form of metabolic activation, which may limit their utility. Chemical complexes that contain nitric oxide and from which NO is released spontaneously at a controlled rate have been described by investigators at the National Cancer Institute. These complexes may prove useful for therapeutic applications where organic nitrates are not effective.[14]

Induced Nitric Oxide and Endothelial Cell Damage

Although endothelial function and NO formation are impaired in hypertension and other coronary artery diseases, the induction of NO production in these situations seems to be harmful rather than helpful. According to Bassenge, "induction of experimentally impaired endothelial NO production (a) suppresses tissue perfusion, (b) results in myocardial ischaemia . . . and (c) can induce hypertension . . ."[5] In their review, Moncada et al. noted that inducible NO synthase is likely to play a role in pathologic vasodilatation and tissue damage.[4]

Palmer et al., concerned with the role of NO in endothelial cell damage, considered the proposition that the expression, but not the activity, of the inducible form of NO synthase is inhibited by glucocorticoids such as dexamethasone and hydrocortisone and that this action is distinct from that of arginine analogs such as L-NMMA, which are specific inhibitors of both constitutive and inducible NO synthases. Using these inhibitors, they examined whether endothelial cells derived from pigs are damaged by the NO synthesized as a consequence of the expression of the inducible NO synthase.[15]

Incubation of vascular endothelial cells with bacterial endotoxin, which induces the expression of NO synthase, led to an increase in cell death. Cytotoxicity was not affected by hemoglobin, suggesting that the NO synthesized in response to endotoxin exerts its effect directly without being released into the extracellular space. Cytotoxicity was inhibited both by L-NMMA and by dexamethasone and hydrocortisone and attenuated in the absence of L-arginine. These findings provide more evidence for an NO-dependent cytotoxicity in endothelial cells, mediated by NO produced by an inducible NO synthase. The investigators conclude: "That the vascular endothelium releases NO constitutively for physiological purposes and also expresses NO synthesis as part of the host defense mechanism exemplifies the diverse role of NO as a biologically active molecule."[15]

Nitric Oxide and Inotropy

Proinflammatory cytokines (e.g., tumor necrosis factor, certain interleukins, etc.) are a class of peptides that are synthesized and released locally by macrophages, leukocytes, and endothelial cells in response to injury. Infiltration by leukocytes and macrophages and the subsequent release of proinflammatory cytokines may be the cause of the myocardial depression that often follows reperfusion of ischemic myocardium. In support of this hypothesis, Finkel et al. have shown that the addition of recombinant hu-

man tumor necrosis factor (TNF), interleukin-6 (IL-6), or interleukin-2 (IL-2) to a tissue bath containing hamster left ventricular papillary muscles results in a concentration-dependent, reversible negative inotropic effect.[16]

Cytokines increase the amount of NO in noncardiac tissue by transcription of an inducible NO synthase.[17] The physiologic effects of cytokines in noncardiac tissue are blocked by L-NMMA, and the effect of L-NMMA is reversed with L-arginine, suggesting that cytokine activity is mediated by NO.[18] Finkel et al. now have shown that the same applies to the negative inotropic effects of cytokines on cardiac tissue. In fact, the addition of arginine to a bath containing TNF and L-NMMA resulted in a more pronounced negative inotropic effect than that seen with TNF alone. Perhaps L-arginine enhanced the negative inotropic effect of TNF by providing additional substrate for NO production.

Finkel et al. concluded that "the observed inotropic effects of pro-inflammatory cytokines raise the possibility that they participate in reversible, postischemic myocardial depression ("stunning"). Myocardial stunning frequently occurs after cardiopulmonary bypass and may complicate successful recovery from cardiac surgery."[16]

Finkel et al also reported that they had found elevated concentrations of IL-6 in bronchoalveolar fluid from patients after cardiopulmonary bypass. Finkel et al. added that "these preliminary observations in patients support the clinical relevance of their findings with the Syrian hamster papillary muscle preparation. Thus, the regulation of proinflammatory cytokines and myocardial NO synthase may provide new therapeutic strategies for the management of cardiac patients."[16]

Nitric Oxide and Septic Shock

Septic shock is shock associated with massive infection, most commonly infection with gram-negative bacteria. It is thought to result from the actions of endotoxins or other products of infectious agents on the vascular system, causing large volumes of blood to be sequestered in the capillaries and veins. The principal cardiovascular features of septic shock are sustained vasodilation, hypotension, and a lack of response to vasoconstrictors. In animals, endotoxin and cytokines such as interleukin-1 (IL-1) and TNF reproduce many of the cardiovascular features of septic shock. These effects appear to be mediated by nitric oxide.

Nava et al. have shown that L-NMMA at a dose of 30 mg/kg can prevent endotoxin shock in rats, presumably by inhibiting the induction of NO. The degree of protection, however, was markedly dose dependent. No protection was found when a 3-mg/kg dose of L-NMMA was given, whereas 300 mg/kg L-NMMA accelerated and enhanced the decrease in blood pressure. This suggests that high doses of L-NMMA inhibit both the constitutive and inducible NO synthase enzymes. The investigators concluded that "NO synthase inhibitors may be helpful in the treatment of hypotension associated with sepsis or the therapeutic use of cytokines, but complete inhibition of endogenous NO synthesis may be counterproductive." They also proposed that "Higher doses of L-NMMA to achieve greater inhibition of endogenous NO synthesis may need to be accompanied by an NO-generating vasodilator."[19]

Petros et al. have described two patients with life-threatening septic shock who had not responded to intravenous fluid replacement and treatment with dopamine and norepinephrine infusions. In both patients, intravenous injections of L-NMMA, .3 or 1.0 mg/kg, caused a rapid but short-lived increase in systolic, diastolic, and mean arterial pressures (MAP) with increased systemic vascular resistance. Mean arterial pressure was increased by 12 mm Hg after the lower dose and by 23 mm Hg after the higher dose of L-NMMA. One patient also received an injection of N^G-nitro-L-arginine methyl ester (L-NAME) 150 μg/kg, another NO synthase inhibitor. This was followed by a striking increase in MAP from 84 to 102 mm Hg. The authors concluded that "These findings indicate than NO synthase induction contributes to the pathogenesis of septic shock and that inhibition of NO synthase may represent a novel therapeutic option."[20]

Using a higher dose of L-NMMA, Geroulanos et al. also reported success in treating a patient with septic shock accompanied by severe hypotension and multiple organ failure. Less than three minutes after an intravenous injection of L-NMMA 7 mg/kg blood pressure was normal for about 25 minutes and then decreased. The relapse was successfully treated with an infusion of norepinephrine.[21]

Nitric Oxide and Secondary Hypotension

Valence and Moncada recently proposed that the cardiovascular changes that frequently develop in patients with cirrhosis are the consequence of low-grade endotoxemia and associated induction of NO synthase.[22] Preliminary evidence in support of this hypothesis has been presented by Midgley et al.[23] They described a patient with severe hepatic failure and hypotension who was given a bolus injection of methylene blue and responded with an increase in blood pressure that persisted for more than 60 min-

utes. Methylene blue reverses vasodilation by blocking the stimulation of guanylate cyclase by nitric oxide.

An accompanying editorial suggested that "similar strategies may likewise become a useful adjunct to the therapeutic use of cytokines such as interleukin-2, which are normally restricted because of the concomitant hypotension caused by induction of NO-synthase and overproduction of NO."[24] An international trade journal reported that an investigational new drug (IND) application has been submitted to the Food and Drug Administration (FDA) to study the use of L-NMMA in cancer patients treated with IL-2; initial clinical trials will establish safety followed by studies to evaluate L-NMMA's role in managing IL-2–associated hypotension, which often prevents the administration of sufficient doses of IL-2 to patients with renal cancer.[25] Such patients also may benefit from pretreatment with glucocorticoids, "which inhibit the expression rather than the activity of the inducible NO synthase without affecting the constitutive enzyme . . ."[25]

Nitric Oxide and Pulmonary Hypertension

With the understanding of the physiologic role of NO came the idea that gaseous NO administered by inhalation might be useful in the treatment of pulmonary hypertension. A concern when considering inhaled NO is that it reacts with oxygen to form nitric and nitrous acids, both of which can damage the lungs. Fortunately, NO combines only very slowly with oxygen, and no adverse effects of inhaled nitric oxide have been seen in laboratory animals even with concentrations as high as 100 ppm.[22]

Frostell et al. have reported that inhalation of a gas mixture containing 40 to 80 ppm NO could reverse the acute pulmonary vasoconstriction induced by severe hypoxia in conscious lambs. Unlike systemically administered vasodilators, which affect both the pulmonary and the systemic circulation, NO exerts a selective vasodilating effect on the pulmonary vasculature because hemoglobin scavenges it rapidly and prevents systemic effects.[26]

More recently, Fratacci et al. described a study in which NO was inhaled by 16 conscious lambs in an attempt to reduce the increase in pulmonary artery pressure and pulmonary vascular resistance induced by a thromboxane analog or the endogenous release of thromboxane that occurs during the neutralization of heparin anticoagulation by protamine sulfate. Inhaling >40 ppm NO during a continuous infusion of the thromboxane analog reduced pulmonary artery pressure to a normal level without affecting systemic blood pressure. Nitric oxide was also effective in reducing peak pulmonary artery pressure after the heparin–protamine reaction, but relatively high levels of nitric oxide were required.[27]

The investigators concluded that "Inhaled NO is a selective pulmonary vasodilator that can prevent thromboxane-induced pulmonary hypertension . . . and can do so without causing systemic vasodilation . . . NO inhalation represents an alternative approach to treating pulmonary hypertension associated with the heparin-protamine reaction as well as other acute and reversible causes of pulmonary hypertension."[27]

Clinically, pulmonary hypertension is a hemodynamic abnormality seen in a variety of acute and chronic pulmonary disease states. Regardless of cause, "the increased pressure in the pulmonary circulation results in a progressive inability of the right ventricle to sustain its output and frequently leads to right ventricular failure and death. Interventions that reduce right ventricular afterload, thereby improving right ventricular function, have long been sought, and many different vasodilator agents have been used in an attempt to achieve such a goal. The optimal vasodilator would selectively dilate the pulmonary vasculature without altering systemic vascular resistance. Unfortunately, no selective or even highly preferential pulmonary vasodilator agents—until now—had been identified."[28]

The clinical application of inhaled NO moved one step closer to reality as a result of a report from investigators at Cambridge who administered air containing 40 ppm NO to eight patients with severe pulmonary hypertension, 10 cardiac patients with normal values of pulmonary vascular resistance, and 10 healthy subjects, and compared its effects with those of an intravenous infusion of prostacyclin. Both NO and prostacyclin induced a rapid decrease in pulmonary vascular resistance in the patients with pulmonary hypertension. Although systemic vascular resistance decreased substantially after prostacyclin in patients with pulmonary hypertension, inhaled NO had no effect on systemic vascular resistance in patients or volunteers. The effects of NO were sustained and reversible, and patients were not able to distinguish between the air/NO mixture and air alone.[29]

More recently, Roberts et al.[30] and Kinsella et al.[31] reported on the effectiveness of inhaled NO in persistent pulmonary hypertension of the newborn. Both studies showed that inhalation of NO resulted in a rapid improvement in oxygenation without affecting systemic blood pressure. Kinsella et al. found that doses of NO as low as 10 to 20 ppm were effective. These findings are particularly important because in newborns, in contrast to adults, short-term

reduction in pulmonary vascular resistance often is sufficient to resolve persistent pulmonary hypertension.

Based on the findings of the preclinical and clinical studies, it would seem that inhaled NO is both a selective and effective pulmonary vasodilator. It also seems likely that therapeutic inhalation of NO will provide a new clinical strategy for the management of patients with pulmonary hypertension and patients with adult respiratory distress syndrome.

Nitric Oxide Synthesis in Chronic Renal Failure

As noted, the synthesis of NO can be inhibited by some analogs of arginine, including L-NMMA. Inhibition of NO synthesis with L-NMMA in laboratory animals leads to hypertension[32] and impaired immune function.[33]

Methylated arginines such as L-NMMA and dimethylarginines (DMAs) are naturally occurring substances. Finding that concentrations of DMAs, in the forms of asymmetric DMA (ADMA) and symmetric DMA (SDMA), were ten times greater than that of L-NMMA in human plasma, Vallance et al. were prompted to study the possible role of DMAs as endogenous inhibitors of the L-arginine to NO pathway in healthy human subjects and in patients with chronic renal failure, a condition associated with hypertension and impaired immunity.[34]

Mean concentration of DMAs in plasma was 1.12 μmol/L in six healthy subjects but increased to 8.7 μmol/L in nine men with end-stage chronic renal failure who required regular dialysis. Dimethylarginine concentrations increased in proportion to serum creatinine. Furthermore, right after hemodialysis, when serum creatinine levels decreased by 30 to 50%, the concentration of DMAs decreased by 40 to 50%.

In vitro, synthetic and endogenous ADMA (extracted from urine) inhibited macrophage and vascular NO synthase, whereas SDMA did not. Infusions of 2 to 16 μmol/minute ADMA into the brachial artery of healthy subjects caused a dose-dependent decrease in forearm blood flow. This effect was significantly attenuated by an infusion of L-arginine.

The findings reported by Vallance et al. indicate that circulating methylated arginine analogs can inhibit NO synthase and suggest the existence of endogenous mechanisms to regulate NO synthesis. Endogenous ADMA usually is excreted unchanged in the urine, but in patients with chronic renal failure plasma ADMA concentrations increase to levels sufficient to inhibit NO synthesis. Vallance's report closes with the question: "Could inhibition of NO synthesis as a result of ADMA accumulation be a common mechanism whereby all causes of renal failure predispose to hypertension and impaired host defence against infection?"

Nitric Oxide and the Gastrointestinal Tract

According to Snyder, NO synthase occurs in the neuronal network in all regions of the gastrointestinal (GI) tract. "These neurons mediate the physiologic relaxation of the gut that participates in the normal peristaltic activity of digestion."[2]

Administration of *E. coli* lipopolysaccharide, a powerful endotoxin, in the rat causes acute intestinal damage, accompanied by marked increases in the intestinal formation of platelet-activating factor (PAF), a membrane-derived proinflammatory phospholipid.[35] Platelet-activating factor has been implicated as a key mediator in the pathologic effects of endotoxin, producing hypotension, profoundly increasing vascular permeability, activating inflammatory cells, and inducing GI damage.[36]

There is some evidence that nitric oxide plays a protective role against endotoxin-induced acute intestinal damage. N^G-monomethyl-L-arginine, enhances the acute damage and the increase in vascular permeability induced by endotoxin,[37] and S-nitroso-N-acetyl-pencillamine (SNAP), which spontaneously generates NO, attenuates both endotoxin-induced intestinal damage and its enhancement by L-NMMA.[38] These findings prompted Boughton-Smith et al. to study in more detail the possible protective effects of NO against PAF-induced intestinal damage.[39]

Studies in the rat using radiolabeled human serum albumin showed that SNAP inhibited PAF-induced gastrointestinal plasma leakage, a measure of the initiation of vascular damage, in a dose-dependent manner. N^G-monomethyl-L-arginine substantially potentiated GI damage and plasma leakage induced by *E. coli* endotoxin, but had no effect on that induced by intravenous infusion of PAF. Because PAF is a mediator of endotoxin-induced intestinal damage in the rat, these findings suggest that endogenous NO protects against a component of endotoxin-induced damage that is not PAF dependent. Other vasoactive mediators such as thromboxane may be released by endotoxin and synergistically interact to induce the GI damage. Endogenous NO therefore may counteract the actions of thromboxane and other mediators.

The investigators concluded that endogenous NO may have a protective role and the potential to prevent microvascular injury in the GI tract that can be mimicked by generators of NO such as SNAP. "The protection afforded by endogenous NO may, how-

ever, be dependent on the nature of the inflammatory stimulus used to induce gastrointestinal damage." [39]

Nitric oxide also provides protection against ethanol-induced gastric mucosal injury. Quintero and Guth found that gastric mucosal blood flow was higher in rats with uremia, induced by subtotal nephrectomy, than in control rats. The investigators suggested that this difference may be the result of local vasodilation in uremic rats, mediated by EDRF. They also found that continuous intragastric perfusion with 40% ethanol produced significantly less gastric damage in uremic than in control rats. Pretreatment of uremic rats with an inhibitor of NO biosynthesis decreased gastric mucosal blood flow and heightened the local toxicity of ethanol. The NO inhibitor was without effect on mucosal blood flow or ethanol toxicity in control rats. The investigators concluded that "in the uremic rat, gastric hyperemia, mediated by increased endothelium-derived nitric oxide, attenuates ethanol-induced gastric mucosal injury." [40]

More recently, investigators from Brussels studied the role of nitric oxide in infantile hypertrophic pyloric stenosis (HPS), a common infantile disorder occurring within a few weeks of birth, characterized by enlarged pyloric musculature and gastric-outlet obstruction. In light of recent information concerning the occurrence of NO synthase in the neuronal system throughout the GI tract and the possible importance of NO in GI relaxation, Vanderwinden et al. set out to study the distribution of NO synthase in the pylorus of normal infants and children and of infants with HPS, using histochemical techniques to detect an enzyme called NADPH diaphorase, now recognized to be a nitric oxide synthase.[41]

The investigators reported that in all pyloric tissue samples NO synthase activity was restricted to the enteric nervous system and blood vessels. In the pyloric tissues from the control infants and children, intense enzyme activity was found in the nerve fibers of the circular musculature, in the neurons and nerve bundles of the myenteric plexus, and in some nerve fibers of the longitudinal musculature. In the pyloric tissues from patients with infantile HPS, the enteric nerve fibers were enlarged and distorted and did not contain NO synthase activity, whereas the activity in the myenteric plexus and the longitudinal musculature was preserved.

Vanderwinden and her colleagues concluded that their findings "confirm that the peptidergic inhibitory innervation of the circular muscular layer is decreased in infantile hypertrophic pyloric stenosis. Moreover, since nitric oxide has an important role in the relaxation of enteric smooth musculature, these results suggest that the nitric oxide synthase in the enteric nerves of the smooth musculature is also involved in the relaxation of the pylorus and that the lack of this enzyme in infantile hypertrophic pyloric stenosis may account for the defect in relaxation of the pyloric sphincter—the old concept of pylorospasm—and leads to the clinical syndrome of gastric-outlet obstruction." [41]

Nitric Oxide in the Nervous System

Moncada et al., in their extensive review of the literature, pointed out that neurotransmission by agents such as glutamate dramatically increases cGMP levels in the brain, particularly in the cerebellum. This effect is mediated by NO, which activates guanylate cyclase. Glutamate turns on N-methyl-D-aspartate (NMDA) receptors, resulting in an increase in NO synthase activity and converting arginine to NO. Inhibitors of NO synthase block the NMDA-induced elevation of cGMP.[4]

Moncada et al. also noted out that "the L-arginine: NO pathway may also play a role in the pathology of the central nervous system. The Ca^{2+} influx that accompanies prolonged NMDA receptor activation is associated with degeneration of the neurons. It is likely excessive NMDA receptor activation, with the consequent increase in Ca^{2+}, contributes to glutamate neurotoxicity by enhanced production of NO." [4]

Lancaster has put forward a provocative hypothesis relating NO to the neurotoxicity and seizure activity associated with alcohol withdrawal in certain people, noting that "alcohol interacts with the glutamate-NMDA receptor by blocking the action of neuronal ion channels, acutely blocking excitotoxicity. However, the chronic effect of alcohol involves a compensatory increase in the numbers of ion channels and sensitivity of the neurons to calcium ions . . . Thus, it is possible that glutamate excitotoxicity . . . powered by nitric oxide, plays a central role in the neurotoxic events that may accompany alcohol withdrawal, ischemia, and stroke associated with chronic alcohol use." [42]

Lancaster also pointed out that "the cerebellum, which is disturbed by alcohol exposure during development and in adulthood has the greatest concentration of nitric oxide synthase. Other brain areas where high nitric oxide synthase activity is observed also are influenced by the actions of alcohol . . . Alcohol-induced disturbances in these brain areas are expressed as changes in learning, memory, vision, tolerance, and drinking behavior." [42]

Clark et al. have offered a hypthesis that nitric oxide plays a central role in cerebral malaria and in conditions clinically similar to cerebral malaria,

such as heat stroke and recovery from major surgery.[43] Although the mechanism of human malarial coma remains unknown, some believe that NO from blood-vessel walls, where it is generated by endothelial and smooth muscle cells stimulated by TNF, diffuses through the blood-brain barrier and transiently disturbs CNS function.[44] Functional changes could include inhibition of glutamate-induced calcium entry, reduced activity of the calcium-dependent NO synthase, and thereby, reduced NO formation in post-synaptic neurons. This mechanism would explain why patients recovered from cerebral malaria do not have a high frequency of residual neurologic deficit and why increased serum levels of TNF correlate with severity of malaria, including the severity of cerebral symptoms.[45]

According to Clark et al., the changes in mental status during heat stroke or recovery from major surgery are clinically similar to those seen during cerebral malaria. They are also associated with high circulating concentrations of cytokines (i.e., TNF, IL-1) that can induce NO generation in vascular walls.[43] Similar neurologic changes are seen after immunotherapy with TNF[46] or TNF-inducing cytokines such as IL-2.[47] Hibbs et al. have shown in human subjects that an infusion of IL-2 results in dose-dependent increases in the formation of NO.[48]

Nitric oxide may also be a key factor in a wide range of drug intoxications affecting cerebral function. For example, the unconsciousness induced by general anesthetics is a coma without subsequent neurologic deficit. Ethanol-induced depression of CNS function, culminating in coma after a large intake, is often confused with cerebral malaria in countries where the incidence of malaria is high. In addition to alcohol excess, opioid narcosis can also be clinically very similar to cerebral malaria. Clark et al. point out that "general anesthetics, ethanol, and perhaps morphine and ammonia share the characteristic of being able to inhibit glutamate-induced entry of calcium into post-synaptic cells. Such inhibition would reduce the activation of nitric oxide synthase, and thus nitric oxide generation, through which glutamate-induced excitatory synaptic activity is thought to cause increases in the level of neuronal cGMP."[43]

Nitric Oxide and Male Impotence

Penile erection is thought to involve neuronally mediated relaxation of the blood vessels and smooth muscle that constitute the corporus cavernosum. Failure to maintain an erection has been effectively treated by direct injection into the penis of nitroglycerin or nitroprusside, both of which are NO donors. These clinical observations stimulated great interest in the role of nitrovasodilators in penile erection and, as a result, there is now strong evidence that NO is the physiologic neuronal chemical mediator of erection.

To better understand the role of NO in penile erection, Burnett et al. carried out several studies including immunohistochemical staining of rat penile tissue with an antiserum highly selective for NOS. They found that the enzyme was localized to rat penile neurons innervating the corporus cavernosum and to neuronal plexuses in the adventitial layer of penile arteries. Burnett et al. also found that small doses of L-nitroarginine, a potent and selective inhibitor of NO synthase, markedly diminished electrophysiologically induced penile erections and that injections of L-arginine partially reversed the effects of L-nitroarginine. They went on to state that the "selective localization of NOS in penile neurons that subserve erection, as well as the ability of NOS inhibitors to block physiologic erection selectively, potently, and completely, imply that NO is the major if not sole neuronal mediator of erection." [49]

Burnett et al. also pointed out some clinical implications of the involvement of NO in erection. "Priapism, a condition of painful, prolonged erections unassociated with sexual arousal or desire, occurs in several clinical situations, including as many as 40% of patients with sickle cell anemia. NOS inhibitors such as nitroarginine might have therapeutic utility in priapism." [49] Still another clinical implication is impotence in adult men.

Impotence is a major clinical problem. The National Center for Health Statistics estimates that in the United States alone, 10 million men suffer from dysfunction of penile erections. Rajfer et al., who studied the pathophysiologic role of NO in male impotence, pointed out that "in the majority of patients, abnormal vascular responsiveness is the underlying cause of impotence, and failure to retain blood within the sinusoids is the most common cause of vasculogenic impotence. Filling of the sinusoidal spaces compresses the outflow venules against the relatively rigid tunica albuginea, causing engorgement of the corpus cavernosum with blood. Thus, failure of penile erection could be due to impaired relaxation of the smooth muscle of the corpus cavernosum."[50]

Rajfer and his colleagues studied strips of corpus cavernosum tissue obtained from 21 men in whom penile prostheses were inserted because of impotence. They found that electrical stimulation of the tissue caused a marked relaxation of the corpus cavernosum. This response was inhibited by N-nitro-L-arginine and N-amino-L-arginine, both of which are

known to inhibit the biosynthesis of NO from arginine. Excess L-arginine largely reversed these inhibitory effects.

The investigators also found that the addition of SNAP, a source of NO, caused rapid and complete relaxation of the corpus cavernosum. The relaxation in response to electrical stimulation or NO was enhanced by M&B 22,948, an inhibitor of cGMP phosphodiesterase. Relaxation of the corpus cavernosum also was inhibited by methylene blue, an inhibitor of cGMP synthesis. Based on their findings, Rajfer et al. suggested that "interference with the L-arginine pathway could be one cause of impotence that is treatable by the administration of direct-acting vasodilators." [50]

The media viewed Rajfer's report with some interest. Blakeslee, writing in the *New York Times*, noted that the findings may lead to new kinds of treatment like a removable skin patch containing "nitric oxide donors" that could be placed on the penis to bring on an erection.[51] Ignarro, in a commentary, observed that "future strategies for the treatment of impotence should include the development of new NO-donor drugs that can be applied locally to the penis by patch, ointment, or controlled-delivery devices."[52]

A recent commentary in *Lancet* noted, "involvement of the NO/L-arginine pathway in penile erection explains pharmacologically the aphrodisiac effect of amyl nitrite, which provides a rapidly available source of exogenous NO . . ." The article also pointed out, "in impotent diabetic men, there is impairment of both neurogenic and endothelium-mediated relaxation in penile corporal smooth muscle whereas responses to exogenous donors of NO are preserved. The enhanced alpha 1-mediated constrictor tone and penile flaccidity found in diabetes may be a direct consequence of impaired NO synthesis."[53]

CONCLUSION

The foregoing material has been offered as a response to the question: "What is nitric oxide and why are so many people studying it?" The vitality of scientific inquiry is embodied in the belief that there are still universes of knowledge to explore. The literal explosion of new information about nitric oxide, a simple molecule that has long been under our noses, reaffirms that belief. Small wonder that *Science* at the close of 1992 named nitric oxide as The Molecule of the Year.[54]

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Clinical Correlates of In Vitro *Plasmodium falciparum* Cytoadherence

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To determine whether isolates of *Plasmodium falciparum* have intrinsically different cytoadherent properties and whether these differences contribute to the clinical severity of human falciparum malaria, we studied the cytoadherence to C32 melanoma cells in vitro of 59 parasite isolates from patients with naturally acquired infections in Thailand. Parasitized erythrocytes adhere to these melanoma cells principally via the glycoprotein CD36, which is also expressed on most vascular endothelium. In vitro cytoadherence was significantly greater for isolates from patients with biochemical evidence of severe malaria. The cytoadherent properties of *P. falciparum* parasites may thus be a virulence factor in human falciparum malaria. However, there was no correlation between the degree of in vitro cytoadherence and cerebral symptoms, which suggests that other receptors and/or host factors may be important in the adherence of malaria parasites to cerebral vascular endothelium. The cytokines tumor necrosis factor, interleukin-1, and gamma interferon, which have been implicated in the pathogenesis of cerebral malaria and are known to promote intercellular adhesion in other systems, did not enhance the cytoadherence of *P. falciparum* isolates to C32 melanoma cells.

The central pathological event in human falciparum malaria is the cytoadherence of erythrocytes containing mature stages of *Plasmodium falciparum* to capillary and postcapillary venular endothelia in the deep vascular beds of vital organs (8). This process, termed sequestration, results in alterations in microcirculatory blood flow, metabolic dysfunction, and local toxicity, and, as a consequence, many of the complications seen in severe falciparum malaria (22). Sequestration occurs in all vital organs, although the degree of microvascular packing of infected cells varies considerably. The process is greatest in the brain (8) and is thought to cause coma in cerebral malaria. In human *P. falciparum* infection, parasitized erythrocytes either sequester or are removed from the circulation (principally by the spleen). The balance between sequestration, which allows parasite survival to schizogony, and splenic clearance is a major determinant of the rate of increase and magnitude of the infecting parasite burden. Within this paradigm, increased pathogenicity is associated with either greater cytoadherence or impaired clearance and possibly with a particular pattern of vital organ sequestration.

To determine whether isolates of *P. falciparum* have intrinsically different cytoadherent properties and whether these differences may contribute to the clinical severity of human falciparum malaria, we have studied the cytoadherence of fresh isolates of *P. falciparum* to C32 melanoma cells in vitro. These neoplastic cells share common surface determinants with cultured human endothelial cells for attachment of parasitized erythrocytes (19) and are much easier to propagate in vitro. The effects of the cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1), and gamma interferon (IFN-γ) on cytoadherence were also examined. Circulating TNF levels have been shown to correlate positively with mortality in *P. falciparum* malaria (6), and all three cytokines are involved in intercellular adhesion in other systems (5, 18).

MATERIALS AND METHODS

Patients. We studied 59 consecutive patients with untreated acute *P. falciparum* malaria whose parasites matured in culture in vitro. These were adult Thai patients who either had had a few falciparum malaria attacks in the past or were experiencing their first infection. On admission, a complete physical examination and routine hematological and biochemical tests were performed. *P. falciparum* malaria was confirmed by the demonstration of asexual parasites in peripheral blood smears. Parasite counts (number of parasites per microliter) were estimated by quantitating the number of infected erythrocytes per 1,000 erythrocytes examined on thin smears. Five milliliters of blood was taken for parasite culture before antimalarial therapy was begun. Washed erythrocytes were either put into culture immediately or were resuspended in RPMI 1640 medium and kept overnight at 4°C.

Clinical assessment of severity. Patients were categorized into three clinical groups according to World Health Organization criteria (24). (i) Cerebral malaria: patients with unrousable coma in falciparum malaria (nonpurposive response to painful stimuli or worse) (21). (ii) Severe falciparum malaria: conscious patients who required admission to a hospital and parenteral antimalarial treatment and who had elevated bilirubin (>50 μmol/liter) and aspartate aminotransferase (>50 Reitman-Frankel units/liter) or elevated serum creatinine (>200 μmol/liter). Anemia was not used in

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this classification because of its multifactorial etiology and longer time course. Patients with preexisting renal or liver disease were excluded. (iii) Uncomplicated falciparum malaria: patients with none of the above.

Reagents. The tissue culture medium used for the culture of malaria parasites was RPMI 1640 medium (Flow Laboratories, Ayreshire, Scotland) supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Flow Laboratories), 25 mM $NaHCO_3$ (Flow Laboratories), gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) at 0.15 mg/ml, and 10% (vol/vol) human AB serum (Merseyside Blood Transfusion Service, Liverpool, United Kingdom). For the culture of melanoma cells, heat-inactivated fetal bovine serum (Flow Laboratories, North Ryde, Australia) was used instead of human serum. Recombinant TNF, IL-1, and IFN-γ (Genzyme Co., Boston, Mass.) were aliquoted, stored at −70°C, and used within 6 months of purchase.

Melanoma cell monolayers. Human amelanotic melanoma cells (ATCC CRL 1585, designation C32r; American Type Culture Collection, Rockville, Md.) were cultured and plated onto 22-mm square glass coverslips at 10^5 cells per coverslip as described previously (18). The monolayers were fixed in 1% Formalin (37% [wt/vol] formaldehyde; Fisher Scientific) and stored at 4°C. A new batch was prepared every 4 to 6 weeks. Fresh unfixed melanoma cell monolayers were used in each study of the effect of TNF, IL-1, and IFN-γ on cytoadherence.

Melanoma cell binding assay. The melanoma cell binding assay used was described previously (20). Briefly, infected blood obtained from patients was cultured in vitro for 24 to 48 h until the majority of the parasites were judged morphologically to be at the late trophozoite/early schizont stage. One milliliter of a 2% (vol/vol) suspension in tissue culture medium supplemented with a single batch of AB serum was added to each petri dish containing a fixed melanoma cell monolayer. Parasite isolates which showed above 5% parasitemia were diluted with uninfected group O erythrocytes and tested at at least two dilutions below 5%. The petri dishes were incubated at 37°C in 5% CO_2 for 1.5 h with gentle rocking by hand every 15 min. Following incubation, the coverslips were washed four times with RPMI 1640 medium and then allowed to dry in air, fixed in methanol, and stained with 10% Giemsa for 20 min. Dried coverslips were mounted on glass slides with DPX mountant. Each coverslip was examined by light microscopy under oil immersion by two independent investigators, and the number of parasitized erythrocytes adherent to at least 1,000 melanoma cells was counted along a horizontal and a vertical axis. The mean count for duplicate slides was calculated and expressed as the number of infected erythrocytes per 100 melanoma cells. The coefficient of variation was less than 5% in 80% of the counts and ranged between 5 and 10% in the remainder.

Cytokines. Fresh monolayers were incubated for 4 to 12 h at 37°C with TNF (200 to 2,000 U; 10 to 100 ng/ml), IL-1 (5 to 10 U/ml), and IFN-γ (100 to 1,000 U; 40 to 400 ng/ml). At the end of the incubation, the monolayers were rinsed in RPMI 1640 medium, after which 1 ml of a 2% infected cell suspension was added to each dish. The binding assay was carried out as before.

Statistical analysis. Cytoadherence of parasites from the three patient groups was compared by using the Kruskal-Wallis one-way analysis of variance with multiple comparisons between groups. Correlations were assessed by Spearman's rank correlation coefficient.

RESULTS

P. falciparum parasites obtained from the peripheral blood of patients varied in the stage of ring form development. To ensure that all isolates were tested at a similar stage of maturation, parasites were cultured in vitro for variable periods of time (24 to 40 h) and examined microscopically every 6 to 8 h until they reached the pigmented trophozoite/early schizont stage. Once this stage of development was reached, there was a period of approximately 8 to 12 h during which cytoadherence remained stable.

Cytoadherence and parasitemia. The degree of cytoadherence of different parasite isolates varied considerably and correlated significantly with the admission parasitemia of the patient ($r = 0.755$, $P < 0.001$, $n = 59$). In preliminary experiments, serial dilutions of six isolates with normal group O erythrocytes were tested (Fig. 1). The tight linear relationship ($r = 0.99$ to 1.00) demonstrated between cytoadherence and parasitemia for all six isolates, which showed initial parasitemias of 2.4 to 10.6%, validated the process of testing parasite isolates either at their original parasitemia level or diluted to a parasitemia level within this range. Cytoadherence was then normalized (corrected binding) by dividing the "raw" binding by the percent parasitemia at the time of the binding assay so that all isolates were compared at an extrapolated value at 1% parasitemia. When corrected binding was plotted against admission parasitemia (Fig. 2), a much weaker association ($r = 0.286$) was demonstrated which appeared to be weighted heavily by three outlying points.

Clinical correlates. The cytoadherence of *P. falciparum* isolates to C32 melanoma cell monolayers from different patient groups is shown in Fig. 3. The clinical and laboratory features of the three categories of patients are summarized in Table 1. The preponderance of male patients reflects the epidemiology of malaria in Thailand in that transmission is mainly of the focal, "forest-fringe" type and men are exposed for occupational reasons. The majority of the patients ($n = 33$) had uncomplicated malaria, while 18 had evidence of organ dysfunction other than cerebral malaria. Eight met the criteria for cerebral malaria, of whom seven also had evidence of other vital organ dysfunction. There was marked variation in the corrected binding, confirming that fresh parasite isolates from patients with naturally acquired infections have diverse cytoadherent properties. The median corrected binding of parasite isolates from patients with uncomplicated malaria (42 infected erythrocytes [IRBC] per 100 melanoma cells) was significantly lower than that of isolates from conscious patients with severe malaria (85 IRBC per 100 melanoma cells; $P < 0.001$). In contrast, the median corrected binding of isolates from patients with cerebral malaria (45 IRBC per 100 melanoma cells) was similar to that of isolates from patients with uncomplicated malaria ($P > 0.05$) and was significantly lower than that of isolates from patients with severe malaria but without cerebral involvement ($P < 0.05$). Pooling of data for both severe groups still gave a median corrected binding of 73 IRBC per 100 melanoma cells, which was significantly higher than that of isolates from patients with uncomplicated malaria ($P < 0.001$).

Effects of cytokines on cytoadherence. Pretreatment of melanoma cell monolayers with any of the cytokines singly or in combination for 4 to 12 h did not have any consistent enhancing effect on the cytoadherence of 10 *P. falciparum* isolates (data not shown).

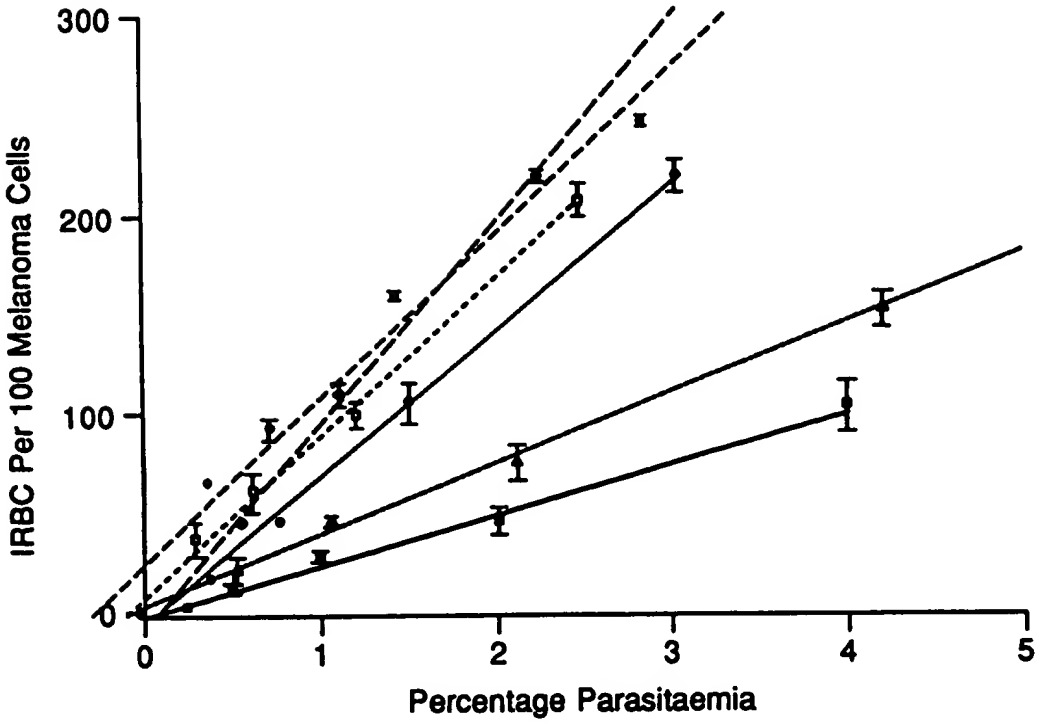

FIG. 1. Effect of trophozoite/schizont parasitemia on cytoadherence of *P. falciparum*. Plotted points are means of duplicate experiments for each of six isolates showing initial parasitemia from 2.4 to 10.6% (r = 0.99 to 1.00). Only data for observations obtained at less than 5% parasitemia are shown.

DISCUSSION

Falciparum malaria is a major cause of death in the tropics. Deep vascular sequestration of parasitized erythrocytes is considered the central pathological event in this infection and, as a result, considerable interest has been focused on the mechanisms underlying this process. The cytoadherence of parasitized erythrocytes to vascular endothelium and certain melanoma cell lines (including the C32 cell line) involves interaction between parasite antigens expressed on the surface of the infected erythrocyte and receptors on endothelial cells. To date, three molecules have been implicated as receptors for parasite ligands: the adhesive glycoprotein thrombospondin (16), the leukocyte differentiation antigen CD36 (12), and intercellular adhesion molecule 1 (ICAM-1) (4). Although both CD36 and ICAM-1 are expressed on C32 cells, cytoadherence to these cells occurs largely through CD36, as shown by inhibition experiments with monoclonal antibodies to the two receptors (2, 4, 12). Furthermore, parasitized erythrocytes can adhere directly to purified CD36 immobilized on plastic (12) or expressed on transfected COS cells (13).

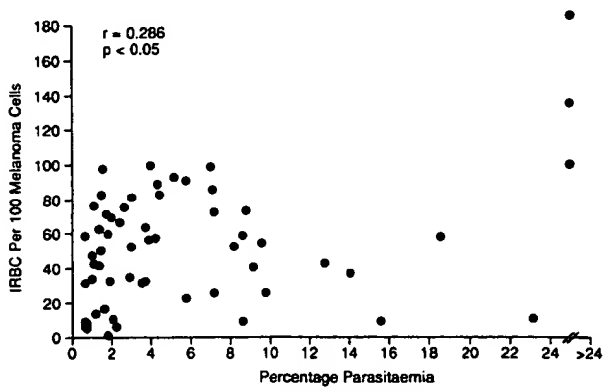

FIG. 2. Corrected binding versus admission parasitemia.

In the present study, cytoadherence of parasitized erythrocytes was found to correlate significantly with parasitemia in vivo. It stands to reason that with more parasitized cells, more would adhere. However, the aim of the current study was to determine if there are intrinsic parasite properties which affect cytoadherence independently of parasitemia and host factors. We eliminated the variation in degree of cytoadherence due to parasitemia per se by comparing the binding of all isolates at the same level of parasitemia (1%). The use of this corrected binding and the fact that in vitro culture largely removes host factors means that we examined only the ability of isolates to cytoadhere.

By so doing, we demonstrated that malaria parasite isolates from natural infections of humans have diverse intrinsic capacities for cytoadherence. The weak association between corrected binding and parasitemia in vivo suggests that higher levels of parasitemia may have resulted from parasite isolates which had bound to a greater extent, allowing a higher multiplication rate. However, the finding should be interpreted with caution as the correlation coefficient was relatively low.

The factors which determine these cytoadherent properties are not fully characterized but may be related to the expression of a family of parasite proteins with different

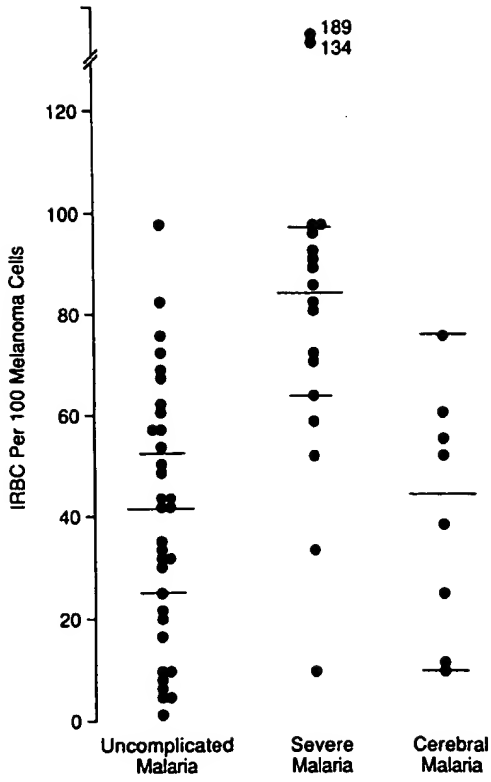

FIG. 3. Comparison of corrected binding for isolates from patients with uncomplicated, severe, and cerebral malaria. The bars indicate the median ± 95% confidence intervals.

molecular weights on the surface of the infected erythrocytes (9). Cytoadherence is associated with the expression of a larger form of the protein (molecular weight, 2.6×10^5) which may contain the adhesive epitopes. Parasites isolated from patients with natural infections may consist of multiple populations expressing different forms of the surface protein, or individual parasites may be capable of producing variant forms of the protein. The degree to which a given isolate cytoadheres in vivo will then depend on various parasite properties and also factors related to the host. These include immune status (20), blood pH (10), and physical determinants such as the shear forces of blood flow within the microvasculature (17).

For *P. falciparum* parasites isolated from conscious patients with severe malaria, there was increased cytoadherence of infected erythrocytes to melanoma cells in vitro compared with isolates from patients with uncomplicated disease. Patients with severe malaria were divided into two groups: cerebral malaria (unrousable coma) or severe disease with raised serum creatinine or bilirubin and aspartate aminotransferase, reflecting impaired renal function and a combination of muscle, liver, and erythrocyte damage. This suggests that the cytoadherent properties of parasites contribute to virulence and supports the hypothesis that cytoadherence to CD36 on vascular endothelial cells is an important pathological mechanism in vivo. Parasites which sequester more readily than others (either earlier in the developmental cycle or to a greater extent) are less likely to be removed by the spleen. This will result in a greater multiplication rate and a more rapid expansion of the parasite burden (23).

The distribution of organ pathology secondary to sequestration in severe malaria is related presumably to the expression and density of receptor molecules on vascular endothelial cells in different tissues, and this in turn determines the distribution of microvascular obstruction, local toxicity, and interference with metabolic function. The pattern of organ dysfunction in severe falciparum malaria varies between patients. Some may become comatose with little evidence of other vital organ dysfunction (21), whereas others may die with acute renal failure, pulmonary edema, hypoglycemia, and lactic acidosis without prior loss of consciousness. In African children, renal dysfunction and pulmonary edema are very unusual in severe malaria (11), whereas these manifestations develop in over half the Thai adults with lethal infections.

By use of monoclonal antibody OKM5, the CD36 antigen has been demonstrated on the vascular endothelium of the

TABLE 1. Clinical and laboratory features of patients with uncomplicated, severe, and cerebral malaria

Parameter	Value for patients with malaria type:		
	Uncomplicated (n = 33)	Severe (n = 18)	Cerebral (n = 8)
Age (yr)	22.7 ± 7.0[a]	25.4 ± 10.1	33.3 ± 13.4
Sex (M:F)	27:6	13:5	6:2
Parasite count (geometric mean/μl)	99,519	279,209	116,396
Haematocrit (%)	35.8 ± 8.5	36.1 ± 8.0	32.0 ± 8.4
Blood urea nitrogen			
mg/dl	17.8 ± 6.7	40.5 ± 35.3	61.9 ± 42.4
mmol/liter	6.3 ± 2.4	14.4 ± 12.6	22.0 ± 15.1
Serum creatinine			
mg/dl	1.2 ± 0.4	2.6 ± 2.7	2.9 ± 1.5
μmol/liter	106.2 ± 35.4	230.2 ± 239.0	256.7 ± 132.8
Total bilirubin			
mg/dl	1.6 ± 1.1	6.4 ± 8.1	11.7 ± 18.9
μmol/liter	27.2 ± 18.7	108.8 ± 137.7	198.9 ± 321.3
Aspartate aminotransferase			
Reitman-Frankel units[b]	33.6 ± 7.8	86.9 ± 64.3	69.4 ± 32.1
μmol/liter	0.56 ± 0.13	1.45 ± 1.07	1.16 ± 0.54

[a] Mean ± standard deviation.
[b] Normal range, 0 to 40 U.

liver, kidney, and lung (7), which may explain the association between binding to melanoma cells in this study and clinical complications involving these organs. However, the demonstration of CD36 on cerebral endothelium from either routine autopsy materials or patients who died of cerebral malaria required the use of a different monoclonal antibody, MAb 8A6 (2), while other investigators have consistently failed to detect the CD36 antigen on cerebral vascular endothelium (3). This suggests that the CD36 molecule is expressed differently on cerebral endothelium or is absent. The variation in molecular expression of CD36 or its absence may explain the lack of association between cytoadherence to melanoma cells in vitro and the clinical syndrome of cerebral malaria found in this study and raises the possibility that CD36 may not be the principal cerebral endothelial receptor for *P. falciparum*-infected erythrocytes. Our results agree with those of the only previous attempt to correlate in vitro measures of cytoadherence and clinical findings, in which no differences in melanoma cell binding were found between *P. falciparum* isolates from Gambian children with cerebral and uncomplicated malaria (10).

Using a line of *P. falciparum* parasites selected for cytoadherence to human umbilical vein endothelial cells, Berendt et al. (4) have demonstrated that some parasitized erythrocytes cytoadhere via (ICAM-1). Whether this receptor molecule has any relevance to the cytoadherence of *P. falciparum* in vivo remains to be determined, but our findings do suggest that receptors other than CD36 may be involved in the cytoadherence of parasitized erythrocytes in human falciparum malaria.

The cytokines TNF, IL-1, and IFN-γ, either singly or in combination, did not enhance the cytoadherence of parasitized erythrocytes to C32 melanoma cells. Since these cytokines act mainly through the induction of receptor molecules on the cell surface, the lack of effect is probably due to the fact that CD36 is already maximally expressed on C32 melanoma cells (1). There is now limited evidence that TNF and IL-1, which upregulate ICAM-1 expression on human umbilical vein endothelial cells (15), may actually enhance the cytoadherence of certain laboratory-adapted parasites to these cells (4). Further studies with isolates obtained from natural infections are needed in order to explain the role of cytokines in the pathogenesis of severe falciparum malaria.

The results of this prospective study have shown that parasitized erythrocytes from patients with severe falciparum malaria have intrinsically greater cytoadherence to melanoma cells in vitro than do those from patients with uncomplicated infections. Thus, the cytoadherent properties of *P. falciparum* may be an important virulence factor. The potential for inhibiting or reversing cytoadherence with antibodies or receptor analogs should be actively explored.

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Antimalarial Drugs Reduce Cytoadherence and Rosetting of *Plasmodium falciparum*

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The in vivo and in vitro effects of antimalarials on cytoadherence and rosette formation were studied in 17 patients with severe and 46 with uncomplicated falciparum malaria. Cytoadherence was increased in severe malaria ($P < .001$). Artesunate and artemether were more potent than quinine in inhibiting both adherence properties. Artesunate was the most rapidly acting drug tested, producing >50% inhibition of both cytoadherence and rosetting in vivo and in vitro within 2 h of drug exposure. Exposure to quinine for ≥4 h in vivo reduced rosetting by >50%, but not cytoadherence. Quinine did not reduce cytoadherence or rosetting significantly in vitro with exposure times of ≤8 h. These results suggest that artemisinin derivatives are more effective than quinine in preventing pathologic processes in parasitized erythrocytes that contribute to microvascular obstruction in severe malaria.

The antimalarial effects of drugs are assessed in vivo by measures such as parasite or fever clearance times and cure rates [1, 2]. In vitro antimalarial activity against *Plasmodium falciparum* is measured conventionally by the inhibition of nucleic acid synthesis (measured by [^{3}H]hypoxanthine uptake inhibition) or maturation to the schizont stage in short-term parasite cultures [3]. These measures may be appropriate for evaluating therapeutic responses in uncomplicated malaria but may not reflect the relative efficacy of antimalarial drugs in preventing pathologic processes in severe falciparum malaria: Lethal events may result from the development of a large parasite burden within a single 48-h asexual life cycle.

P. falciparum parasites induce the expression of erythrocyte surface adhesins that cause infected cells to adhere to vascular endothelium (cytoadherence) and to uninfected red blood cells (RBCs) (rosetting). These adherence properties are considered fundamental to the pathogenesis of severe malaria [3]. They cause sequestration of the parasitized RBCs, which leads to microvascular obstruction and consequently to vital organ dysfunction [4]. Rosetting has been associated specifically with cerebral malaria (i.e., unrousseable coma in severe falciparum malaria) [5]. These adherence properties develop toward the end of the first half of the asexual life cycle (~18–26 h of parasite maturation) and before maximum nucleic acid synthesis. Here we describe the effects of antimalarial drugs on the development of these pathologic adherence properties in vitro and in vivo.

Materials and Methods

Patients. Blood samples were obtained from patients with acute falciparum malaria who were admitted to Pahalpolpayuhasena Hospital or Sangklaburi Hospital, Kanchanaburi, Thailand. Patients with uncomplicated malaria who had taken antimalarial drugs before admission were excluded. Some of the patients were included in studies of antimalarial pharmacokinetics [6].

Management. On admission, a peripheral blood smear was made to confirm the diagnosis of falciparum malaria and to stage parasite development (described in [7]). A full clinical examination was done, and baseline blood samples were obtained for routine hematology, biochemistry, and serum quinine concentration assessments. Antimalarial treatment was started immediately. Patients with uncomplicated malaria were treated with one of the following regimens: oral quinine sulfate (Government Pharmaceutical Organisation, Bangkok; 10 mg of salt/kg, every 8 h for 7 days) with tetracycline (4 mg/kg, every 6 h), $n = 3$; parenteral halofantrine hydrochloride (SmithKline Beecham, Welwyn Garden City, UK; 1 mg/kg intravenously over 1 h, every 8 h for a maximum of 3 doses [i.e., total dose, 3 mg of base/kg]), $n = 5$ (this regimen was part of a preliminary evaluation and has been reported [6]); oral artesunate (Guilin Number 1 Factory, Guilin, China; 4 mg/kg), followed by mefloquine (Lariam; Roche, Basel, Switzerland; 25 mg of base/kg given 24 h later), $n = 33$; or oral artemether (Kunming Pharmaceutical Factory, Kunming, China; 4 mg/kg daily for 3 days) and mefloquine (25 mg of base/kg given 24 h later), $n = 5$.

Patients with severe malaria ($n = 14$) [8], who had not been previously treated with quinine, were given a standard loading

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Patients or their relatives gave fully informed consent. The studies were approved by the Ethical and Scientific Review Subcommittee of the Thai Ministry of Public Health.

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dose of intravenous quinine hydrochloride (20 mg of salt/kg infused over 4 h), followed by maintenance doses (10 mg/kg) every 8 h until they could tolerate oral therapy [8]. Patients already receiving quinine were not given a loading dose. Three patients were treated with intravenous artesunate (Guilin Number 2 Pharmaceutical Factory, Guangxi, China; 2 mg/kg immediately followed by 1 mg/kg every 12 h).

Study design. The objective of the study was to compare antimalarial drug effects on *P. falciparum*–induced erythrocyte adherence in vivo and in vitro. The adherence properties of infected erythrocytes were assessed in terms of cytoadherence to cultured human umbilical vein endothelial cells or of rosetting with uninfected erythrocytes. Studies were done before treatment and at intervals after parasites had been exposed to the antimalarial drug in vivo. Parallel studies were done in vitro: Pretreatment parasites were exposed to the four study drugs (quinine, halofantrine, artesunate, and artemether) under culture conditions, and adherence properties were assessed at the same times as in the in vivo study. Unless stated otherwise, all adherence assays were done after short-term in vitro culture when the control parasites had reached the mature trophozoite-schizont stage [7].

In vivo study. Blood was collected in heparin (10 U/mL) before antimalarial treatment was started (0 h) and at 2, 4, 8, and 18–24 h after treatment had begun, unless indicated otherwise. Patient RBCs were washed twice in 10 mL of RPMI 1640 (GIBCO, Grand Island, NY), pH 7.4, containing 25 m*M* HEPES (*N*-[2-hydroxyethyl] pipecazine-N'-[2-ethenesulfonic acid]), and resuspended and adjusted to 2% hematocrit with RPMI-HEPES medium supplemented with 2 m*M* glutamine, 10% heat-inactivated AB-positive serum, 5 IU of penicillin, and 5 μg/mL streptomycin. The RBC suspension (0.5 mL) was added to a 24-well tissue culture plate (Flow Laboratories, Irvine, UK) and incubated at 37°C in a candle jar for 24–30 h or until the majority of parasites in the drug-free control culture were at the mature trophozoite or early schizont stage of development. Smears were made at frequent intervals to assess parasite maturation. Degree of parasitemia and stage of parasite development were determined by microscopy at the start and end of culture.

To control for the effects of parasitemia on cytoadherence and rosette formation, the infected admission RBCs were diluted to three different levels of parasitemia (0.1–17%, depending on the percentage at admission) with O-positive RBCs from a healthy donor and resuspended at 2% hematocrit in malaria culture medium. The parasites were then cultured as described above, and the relationship between parasitemia and adherence properties was determined for each isolate.

In vitro drug exposure. Heparinized blood that had been drawn on admission was prepared as above. RBC suspensions (0.5 mL at 2% hematocrit) at the patient's presenting parasitemia were incubated with quinine (1 μg/mL, $n = 36$) and with halofantrine ($n = 20$), artesunate ($n = 25$), and artemether ($n = 22$) at 0.25 μg/mL under culture conditions identical to those described above. Drug exposure times were 2, 4, 8, and 18–24 h to coincide with the clinical sampling schedule. At the end of each incubation period, the antimalarial drug was removed by three washes with 4 mL of RPMI-HEPES, pH 7.4. The parasites were then resuspended in RPMI culture medium and incubated 24–30 h or until parasites in the control culture (no drug exposure) were at the late trophozoite or early schizont stage, that is, at equivalent developmental stages to those in the patient with concurrent in vivo exposure to antimalarial drug [7].

Endothelial cell culture. Endothelial cells were obtained from human umbilical veins and cultured in M199 medium (GIBCO, Paisley, UK), pH 7.4, containing 25 m*M* HEPES, 10% heat-inactivated fetal bovine serum, 200 μg/mL endothelial cell growth factor (Biochemical Supplies, Bethesda, MD), 10 U/mL heparin, and 20 μg/mL gentamicin as described [9]. Staining of second-passage cells with antibodies to factor 8, intercellular adhesion molecule–1, vascular cell adhesion molecule–1, E-selectin, and CD36 revealed >98%, 44%, 37%, 37%, and 0 positivity, respectively, confirming that the test cells had retained the characteristics of endothelial cells. Only endothelial cells from passages 2–5 were used in the cytoadherence assays.

Cytoadherence assay. Monolayers of endothelial cells were prepared on gelatin-coated petri dishes and grown 18 h before use. Binding assays were done in parallel for in vivo and in vitro drug-exposed parasite cultures. Parasite cultures were layered onto live endothelial cells and incubated at 37°C for 1 h without agitation [10]. Unbound RBCs were then rinsed with PBS, and the monolayers were fixed with 0.5% glutaraldehyde and washed, dried, and stained with Giemsa and examined by microscopy (100× objective). Three hundred endothelial cells were examined, and the number of infected RBCs bound was counted. Cytoadherence was expressed as the number of infected RBCs bound per 100 endothelial cells. Because the initial studies of the relationship between cytoadherence and parasitemia indicated that the two were correlated in a stationary-adherence assay, all cytoadherence results were normalized to a 1% parasitemia using the correction factors derived from the individual parasitemia-cytoadherence relationships. Because different patients presented with parasites at different stages of development, the drug effects were expressed as percentage inhibition calculated from the ratio of cytoadherence of drug-exposed parasitized RBCs to the cytoadherence of the untreated (control) parasitized RBCs. Circulating infected RBCs obtained from patients before antimalarial treatment were also examined for cytoadherence after being washed but without being cultured.

Rosette formation assay. Rosette formation was assessed at the same times as cytoadherence. A rosette was defined as a central infected RBC bound to two or more uninfected RBCs. Enumeration of rosettes was done immediately at the end of the experiment as described [10], or the cultures were fixed with glutaraldehyde (0.2% final concentration) and rosettes were counted later. We have shown previously that this does not alter the size or number of rosettes [10]. One drop of culture was mounted on a glass slide with a coverslip and examined by light microscopy (100× objective). Two hundred infected RBCs were examined, and the percentage of rosette-forming parasitized erythrocytes was calculated. The rosette formation of drug-exposed parasites and of control (unexposed) parasites was compared and expressed as a percentage. Because there was no correlation between parasitemia and rosetting in the preliminary dilution studies, the results were not normalized. Circulating infected RBCs obtained from patients before treatment were also collected in RPMI-HEPES medium containing heparin (10 U/mL), and rosettes were counted immediately (i.e., without further culture). All other assessments of rosetting were done in the absence of heparin.

Table 1. Mean ± SD (range) values for clinical and laboratory features at admission in patients with severe and uncomplicated falciparum malaria.

	Severe malaria (n = 17)	Uncomplicated malaria (n = 46)
Age (years)	31 ± 13 (16–42)	25 ± 12 (18–62)
Weight (kg)	48.8 ± 15.1 (50–99)	45.1 ± 15.3 (47–64)
Temperature (°C)	37.8 ± 1.3 (36–40)	38.5 ± 1.1 (36.6–39.6)
Fever clearance (h)	56.3 ± 30.1 (0–88)	61.3 ± 34.0 (0–96)
Hematocrit (%)	33.8 ± 4.8 (17–99)	36.4 ± 5.7 (25–51)
White blood cell count/μL	9263 ± 5153 (6000–20,900)	6336 ± 3375 (3800–24,500)
Parasite density/μL*	4.7×10^5 (2.2×10^5–9.9×10^5)	8.9×10^4 (1.9×10^4–1.8×10^5)
PC_{50} (h)	16.7 ± 17.7 (10–43)	14.2 ± 10.6 (6–44)
PC_{90} (h)	23.7 ± 20.7 (26–40)	20.4 ± 15.4 (9–56)
PCT (h)	51.8 ± 41.2 (88–100)	43.9 ± 37.6 (9–132)
Blood urea nitrogen (mg/dL)	40.4 ± 27.1 (8.2–125)	18.4 ± 10.8 (9–132)
Creatinine (mg/dL)	1.9 ± 1.5 (0.7–6.4)	1.2 ± 0.3 (0.9–2.3)
Albumin (g/dL)	3.7 ± 1.8 (1.4–9.9)	3.8 ± 0.6 (2–5.1)
Total bilirubin (mg/dL)	5.3 ± 6.2 (0.2–28.7)	1.7 ± 1.9 (0.1–5.4)
Aspartate aminotransferase (U/L)	96.7 ± 68.9 (13–198)	43.9 ± 25.1 (9–87)
Alanine amino transferase (U/L)	62.5 ± 54.0 (15–304)	31.6 ± 20.2 (4–102)
Alkaline phosphatase (U/L)	62.4 ± 39.2 (12.2–159)	38.5 ± 17.8 (9–99)
Lactate (mmol/L)	8.4 ± 4.6 (1.9–18.7)	2.5 ± 0.9 (1.2–5.5)
Glucose (mmol/L)	11.2 ± 20.5 (2.5–113.5)	6.8 ± 1.5 (4.8–10.2)

NOTE. PC_{50}, PC_{90}, and PCT are times for parasite count to fall by 50% and 90% of admission value and below level of microscopic detection, respectively.
* Geometric mean.

Data analysis. Drug effects on parasites were expressed in terms of percentage inhibition of either cytoadherence or rosetting in comparison with the simultaneously cultured parasites not exposed to the drugs. The maximum inhibition observed was termed E_{max}%, and the time to 50% of maximum inhibition derived from the individual time series was termed ET_{50}.

Data within one group and between the groups of antimalarial drugs were assessed by the Kruskal-Wallis rank test or analysis of variance with the Scheffe post-hoc test. We used the Wilcoxon rank sum test to compare adherence properties between severe and uncomplicated malaria. Correlations were assessed by the methods of Pearson or Spearman.

Results

Table 1 shows patient clinical and laboratory features at admission. Of the 63 patients studied, 11 with severe malaria died; the others recovered fully. Thirty-six patients received artesunate and 5 received artemether (both followed by mefloquine), 17 received quinine, and 5 halofantrine. Serial parasite counts did not reveal any evidence of sequestration reversal with any of the antimalarial treatments (i.e., liberation of erythrocytes containing mature stages of *P. falciparum*).

Cytoadherence and rosette formation of noncultured circulating parasitized RBCs. Most circulating infected RBCs from patients with uncomplicated malaria were synchronous with only ring stages present in peripheral blood samples. These ring stage parasites did not cytoadhere to endothelial cells or form rosettes with uninfected RBCs. However, 9 of the 10 peripheral blood smears from patients with severe malaria in whom this was assessed showed high and asynchronous parasitemias with all stages of development, ranging from ring stages to segmented schizonts. Cytoadherence was determined in 8 cases directly from fresh (noncultured) circulating infected RBCs. Seven isolates cytoadhered readily to endothelial cells (table 2). In addition, 8 of the 10 isolates formed high percentages of rosettes (≥20%) despite the use of heparin (10 IU/mL) as an anticoagulant. The other 2 admission isolates showed few or no rosettes.

Cytoadherence and rosette formation in cultured admission isolates. Adherence properties were assessed in cultured par-

Table 2. Cytoadherence and rosette formation of fresh parasitized red blood cells at hospital admission in patients with severe malaria.

Patient	Parasitemia (%)	Cytoadherence*	Rosetting (%)
1	8.4	ND	25
2	11.4	4	31
3	5.8	55	25
4	20.2	ND	25
5	10.9	4	20
6	19.9	0	1
7	10.0	2	0
8	13.9	30	43
9	14.4	49	44
10	5.0	3	41

NOTE. All patients but no. 8 received quinine before hospital admission.
ND, not determined.
* Expressed as actual no. of parasitized red blood cells binding to 100 endothelial cells.

Table 3. Comparison of in vivo and in vitro antimalarial pharmacodynamics.

	In vivo				In vitro			
	Cytoadherence		Rosetting		Cytoadherence		Rosetting	
Antimalarial	ET_{50} (h)	E_{max} (%)	ET_{50} (h)	E_{max} (%)	ET_{50} (h)	E_{max} (%)	ET_{50} (h)	E_{max} (%)
Quinine	24 (16)*	64 (73)†	4 (7)*	70 (38)†	8 (20)*	87 (44)	24 (20)*	78 (94)†
Halofantrine	6 (4)	61 (21)	1 (0.25)	100 (0)	2 (3)	82 (49)	1 (1)	100 (13)†
Artesunate	1 (0.25)*	99 (0.5)	1 (1)*	100 (0)	2 (2)*	100 (3)†	2 (2)*	100 (1)*
Artemether	6 (5)	90 (25)	4 (6)	100 (0)	8 (10)	96 (25)	4 (6)	90 (44)†

NOTE. Values are median and interquartile data from pooled results from severe and uncomplicated malaria. E_{max} = maximum inhibitory effect, ET_{50} = time until 50% of E_{max} is achieved (derived from individual concentration-effect relationships). Significant differences ($P < .05$) between drugs are shown as * or †: Where there is a difference between 1 drug and others, only that drug is indicated; where difference is between 2 drugs, both are indicated.

asites only when the majority of parasites were at the mature trophozoite or schizont stage. As expected, after ex vivo culture for 24–30 h, the number of parasitized RBCs (PRBCs) bound to endothelial cells showed a positive correlation with the number of mature parasite stages in the blood sample for trophozoites ($r = .73$, $P = .01$; $n = 17$) and for schizonts ($r = .32$, $P = .01$; $n = 41$). Nevertheless there was considerable variation in both cytoadherence and rosetting among admission isolates. The percentage of infected RBCs forming rosettes in both the severe and uncomplicated groups did not correlate with parasitemia or pretreatment cytoadherence.

The median values for cytoadherence but not for rosetting of parasites from severe malaria were significantly higher than from uncomplicated malaria. The respective values were 17 versus 3 (ranges, 0–212 and 0–44) PRBC/100 endothelial cells and 7% versus 6% (ranges, 0–80% and 0–57%; Wilcoxon rank sum test, $P = .001$ and .063).

Effect of antimalarial drugs on cytoadherence and rosette formation of P. falciparum (table 3). There were no differences between parasites from subjects with severe or uncomplicated malaria in the degree of inhibition of cytoadherence and rosetting by any of the drugs used in vitro. In the control cultures, >90% of parasites eventually underwent merogony and reinvasion.

Quinine. All 17 preexposure (control) cultured parasite isolates adhered to live endothelial cells (2–28 PRBC/100 endothelial cells after normalization to 1% PRBC), whereas preexposure cultured parasite rosetting varied between 0 and 43%. Because results from the 3 patients with uncomplicated malaria were similar to those in the severe malaria group ($n = 14$), the data were pooled. Exposure of the parasites to quinine in vivo during treatment did not consistently affect cytoadherence (figure 1). Furthermore, of the 8 patients who said they had received previous quinine treatment, 4 had serum quinine concentrations ≥2.5 μg/mL, but the parasite isolates from all 4 rosetted and cytoadhered. Four hours after quinine treatment, a significant reduction in rosetting ($P = .01$) occurred (figure 1). The in vitro inhibitory effect of quinine on cytoadherence was greater than the in vivo effect, although there was considerable variation among isolates. The median E_{max} and the interquartile range (IQR) in vitro were 87% (44%), and the median ET_{50} was 8 h. In vivo, the E_{max} (IQR) was 64% (73%), and the ET_{50} was 24 h; $P < .05$. The effect on rosetting was also greater in vitro than in vivo (figures 1, 2). The median E_{max} (IQR) was 78% (94%) and the ET_{50} was 24 h in vitro: Respective findings in vivo were 70% (38%) and 4 h (table 3). Overall, there was considerably more interindividual variation in inhibitory effects with quinine than with the other antimalarial drugs tested.

Halofantrine. After culture ex vivo for another 24–30 h, control cultures of only 3 of the 5 isolates showed cytoadherence (4–7 PRBC/100 endothelial cells after normalization at 1% PRBC). All 5 isolates formed low percentages of rosettes (5%–12%). Exposure of parasites to halofantrine for ≥2 h in vivo and in vitro significantly reduced cytoadherence and rosetting, $P = .01$ (figures 1, 2). Halofantrine completely inhibited rosetting in vivo and in vitro with an ET_{50} of 1 h. The inhibitory effects on cytoadherence were considerably less: The median E_{max} inhibition in vivo (IQR) was 61% (21%), and the ET_{50} was 6 h; in vitro these were 82% (49%) and 2 h.

Artesunate. The effects of oral ($n = 33$) and parenteral artesunate ($n = 3$) were similar, and we pooled the results. In vivo exposure to artesunate reduced both cytoadherence and rosetting significantly within 2 h ($P \leq .03$) (figure 1). These effects were more rapid than with in vitro exposure ($P < .05$). The inhibitory effects of artesunate on cytoadherence were almost complete after exposure for 24 h; median E_{max} (IQR) = 99% (0.5%), ET_{50} = 1 h in vivo versus 100% (3%) and 2 h in vitro (table 3). Two hours after drug administration, the median inhibition was 83% in vivo compared with 53% in vitro; $P = .03$ (figures 1, 2). Artesunate was more potent than artemether in inhibiting both cytoadherence and rosetting. In vitro, the differences between the two drugs became statistically significant after 2 and 4 h of drug exposure. As with artemether and halofantrine, the inhibitory effects of artesunate on rosetting were more rapid in vivo than in vitro, but the differences were not significant; median E_{max} (IQR) = 100% (0%), ET_{50} = 1 h in vivo versus 100% (0.7%) and 2 h in vitro (table 3).

Artemether. The in vitro inhibitory activity of artemether on cytoadherence was similar to the effect in vivo: The median

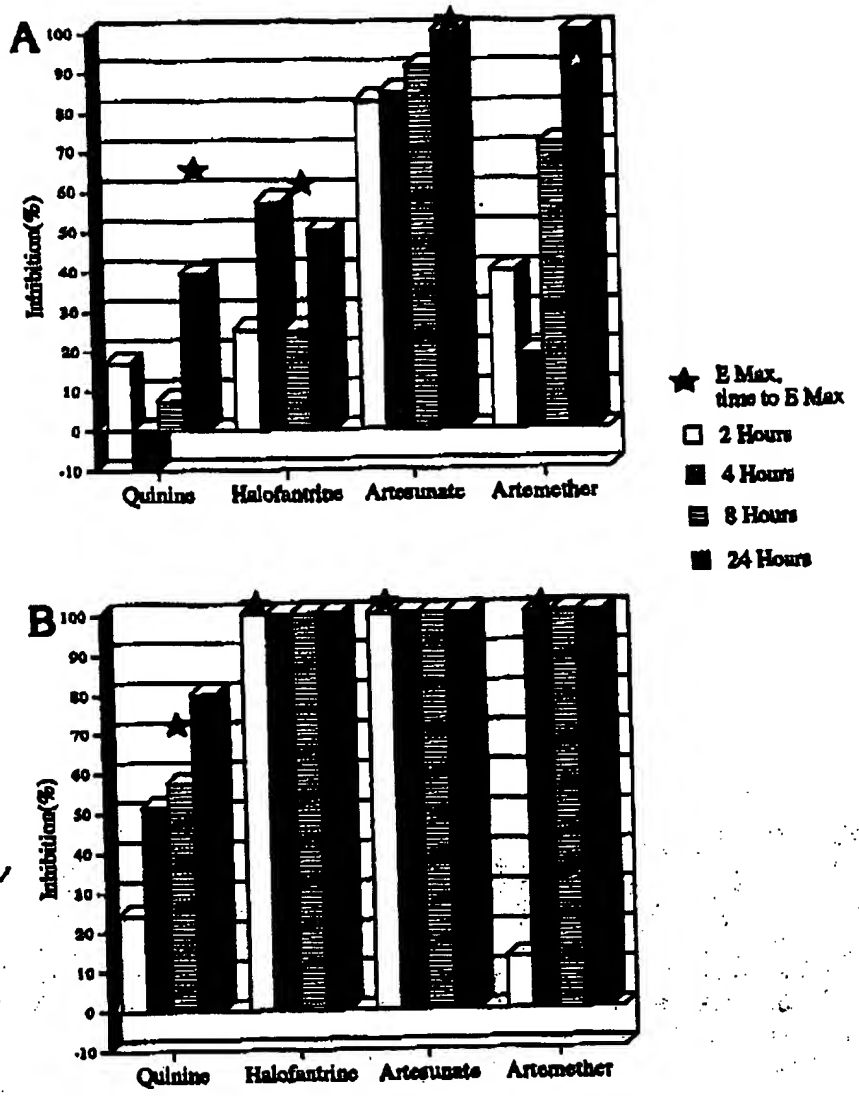

Figure 1. Median inhibition of development of cytoadherence (A) and rosetting (B) with different in vivo exposures to 4 antimalarial drugs. Star, overall median maximum effect (E_{max}), overlies time bar corresponding to median time to E_{max}.

E_{max} (IQR) = 96% (25%), and the ET_{50} = 8 h in vitro versus 90% (25%) and 6 h in vivo. The in vivo effect on rosetting was also not significantly different from the effect in vitro: median E_{max} (IQR) = 100% (0%), ET_{50} = 4 h compared with 90% (44%), 4 h, respectively.

Comparison of effects of antimalarial drugs. The pharmacodynamic effects of the different antimalarial drugs are shown in table 3. Both in vivo and in vitro, artesunate was the most rapidly acting of all the drugs tested and induced >50% inhibition of both rosetting and cytoadherence within 2 h. Artemether was slightly slower; in vitro it was less active than artesunate ($P < .05$). Both in vitro and in vivo, halofantrine was as effective as artesunate in inhibiting rosetting, but in vivo it was less effective than either of the artemisinin derivatives in inhibiting cytoadherence. Quinine was the least active of all the compounds tested both in terms of speed of action and, except for the comparable activity of halofantrine on cytoadherence, it had the lowest maximum inhibitory activity ($P < .05$). Overall, in terms of maximum activity at the concentrations chosen in vitro, artesunate was the most active drug tested; but artemether was more effective in vitro than quinine with drug exposures of 2–8 h ($P \leq .03$). For all four drugs, the E_{max} in vitro and in vivo were correlated: Spearman's rank correlation coefficients were .44 for quinine ($P = .04$), .88 for halofantrine ($P = .02$), .49 for artemether ($P = .17$), and .49 for artesunate ($P = .04$)

Discussion

Although there is extensive information on the efficacy of antimalarial drugs in inhibiting parasite development and multiplication in vitro, there are few data on the effects of antimalari-

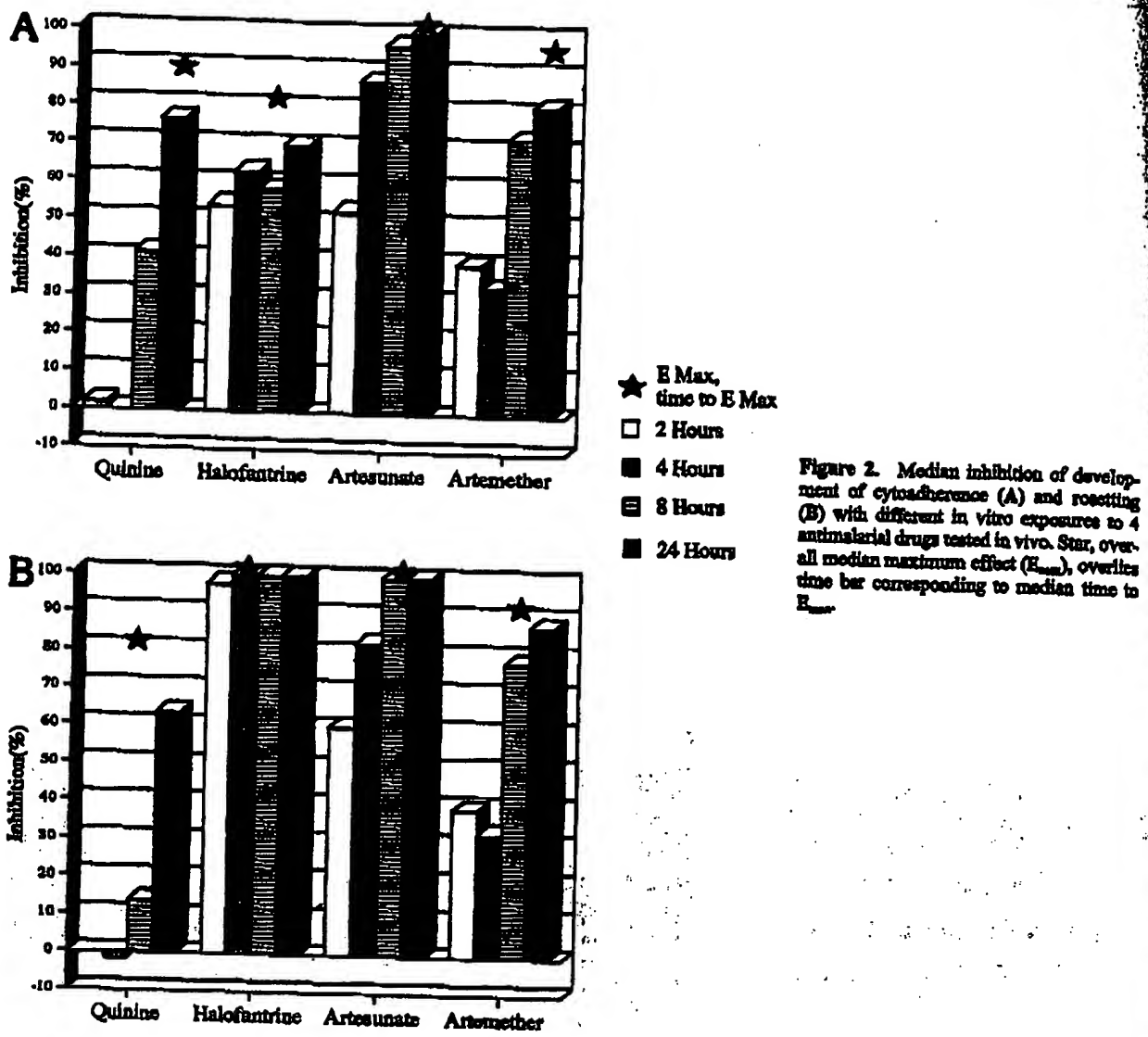

Figure 2. Median inhibition of development of cytoadherence (A) and rosetting (B) with different in vitro exposures to 4 antimalarial drugs tested in vivo. Star, overall median maximum effect (E_{max}), overlies time bar corresponding to median time to E_{max}.

als on pathophysiologic processes in severe malaria [11]. The propensity of *P. falciparum*–infected erythrocytes to adhere to vascular endothelium and other RBCs is thought to be a major factor accounting for the virulence of this parasite [4]. Both of these processes contribute to microvascular obstruction. The potentially lethal syndrome of cerebral malaria is specifically associated with cerebral sequestration [12, 13] and with increased rosetting [5]. In vivo studies of parasite development indicate that the prognosis of severe malaria is related to the sequestered malaria parasite biomass [7]. Although rosetting and cytoadherence occur at the same stage of parasite development, roughly midway through the 48-h asexual life cycle [5, 10], they are mediated by different proteins expressed on the surface of the infected erythrocyte.

The results of this study are consistent with prevention of cytoadherence and rosetting by certain antimalarial drugs, presumably by inhibiting the synthesis and expression of the surface adhesin, rather than reversal of the process once it has developed. Although inhibition of cytoadherence and rosetting were correlated significantly with E_{max} in vitro, the kinetics of inhibitory action on the two processes were different for each antimalarial drug. There were also considerable differences between the antimalarial drugs quinine and halofantrine and the two artemisinin derivatives, artesunate and artemether, in their inhibitory effects. Quinine, the current treatment of choice for severe chloroquine-resistant falciparum malaria, was less effective both in vivo and in vitro than the other three drugs. However, this was not a randomized comparison of drug effects

in vivo, the sample sizes in the artemether and halofantrine groups were small, and the majority of quinine-treated patients had severe falciparum malaria, whereas the majority of artesunate-treated patients had uncomplicated infections.

When antimalarial drug effects are assessed in vitro, a sample of the patient's circulating parasite population, obtained at a single time point, is exposed to a fixed drug concentration under constant temperature conditions and in the relative absence of white cells, drug-binding proteins, and other plasma constituents. In vivo, the population of circulating parasites changes as a result of merogony and sequestration, and several different antiparasitic processes act in concert including fever, antibody concentrations, splenic filtration, active phagocytic parasite clearance, and changing antimalarial blood concentrations. Despite these differences, the results of this pharmacodynamic assessment were similar in vitro and in vivo. Of the four drugs tested, quinine had the least effect on the development of adherence properties and exhibited the greatest variability between isolates. There was no difference in drug effects on parasites from severe or uncomplicated falciparum malaria in vitro, which suggests that the in vivo differences between quinine and the other compounds reflect a difference in pharmacodynamic properties rather than a difference in host or parasite susceptibility. Although halofantrine and the two artemisinin derivatives were considerably more effective in inhibiting parasites from developing the ability to rosette, and the two artemisinin derivatives were both potent inhibitors of cytoadherence, these adherence properties were not abolished completely. This may be explained in part by the differences in parasite maturation in the admission samples. Once adherence properties develop, the drugs do not reverse them. As only one antimalarial concentration was tested, it is not possible to exclude greater effects with higher concentrations. Obviously some or all of the differences between the in vitro and in vivo results could be explained by differences in drug concentrations.

In vitro studies of the stage-specificity of antimalarial action have shown that the artemisinin derivatives have the broadest range of activity on *P. falciparum* protein synthesis [14] and affect younger stages of parasites more than quinine. In contrast, quinine has relatively little effect on protein synthesis before 24 h of parasite development, and production of the proteins involved in cellular adhesion is presumably inhibited relatively little. Thus, these differences in drug effects on the development of parasite adherence probably reflect nonspecific differences in the susceptibility of asexual stage parasites to antimalarials, rather than a specific inhibitory effect on adherence properties. Differential effects on the two adherence properties may be explained by different kinetics of adhesin production and expression. Studies of parasite population dynamics in falciparum malaria also suggest that quinine does not prevent sequestration [15]. At therapeutic concentrations, quinine requires prolonged exposure to parasites to exert maximal effects on parasite clearance [15, 16]. Watkins et al. [17] showed that halofantrine but not quinine or pyrimethamine-sulfadoxine reduces the viability of circulating ring form parasites within the first 24 h of drug exposure in vivo. Despite therapeutic concentrations of quinine and full in vitro sensitivity, parasites continued to mature through the ring stage of development.

In contrast to the relatively weak effects of quinine, artesunate had a rapid inhibitory effect and reduced both cytoadherence and rosetting in vivo within 2 h. These effects were evident before significant parasite clearance (i.e., they did not represent selection of a less adherent subpopulation by the treatment). After 2 h of exposure, artesunate produced a lasting (24 h) inhibitory effect on rosetting that was greater in vivo than in vitro (83% vs. 53%, respectively). Artesunate acted more rapidly than artemether, but after 8 h of treatment, artemether had caught up with artesunate, and thereafter their inhibitory effects were similar. In vitro, artesunate was more potent than artemether in inhibiting both adherence properties. Artesunate is water soluble but is unstable at neutral pH and decomposes readily to its principal biologically active metabolite, dihydroartemisinin (DHA), which is relatively insoluble. Artemether is more stable but is lipophilic and hydrophobic and is intrinsically two to three times less active than DHA [18]. After intravenous injection, artesunate is immediately bioavailable, and it is also absorbed rapidly after oral administration [11]. Hydrolysis to DHA is almost instantaneous. Absorption of artemether is rapid after oral administration [19] but relatively slow after intramuscular injection, and conversion of the parent compound to DHA is also much slower (unpublished data). Thus, both pharmacokinetic and pharmacodynamic factors probably explain the differences between the two artemisinin derivatives.

As shown previously [7], circulating infected RBCs from patients with uncomplicated malaria generally presented with synchronous young ring stage infections that did not rosette and did not cytoadhere to endothelial cells. In contrast, a greater proportion of circulating infected erythrocytes from patients with severe malaria contained more mature parasites and could still cytoadhere, even though 4 patients were treated with quinine before hospitalization (admission serum quinine levels, $\sim \geq 2.5$ μg/mL). Circulating parasitized erythrocytes from severe malaria also maintained their ability to form rosettes despite the presence of heparin (10 IU/mL). This differs from reports of a laboratory *P. falciparum* strain in which rosetting was inhibited by heparin at a much lower concentration [20].

The considerable differences in antimalarial drug effects on the development of cytoadherence or rosetting may be of relevance both to the treatment of severe malaria and in the prevention of vital organ dysfunction in patients with high parasitemias but no other signs of severity [8]. Such patients usually have a predominance of young ring form–infected erythrocytes, and if these can be prevented from developing to the more pathologic mature stages that cytoadhere and rosette, complications may be prevented. Quinine is becoming the most widely used drug worldwide for the treatment of severe falciparum malaria, yet its efficacy in inhibiting cytoadherence and

rosetting is inferior to the artemisinin derivatives. Although these studies were conducted in an area with reduced quinine sensitivity, studies with higher concentrations of quinine [14] or more drug-sensitive parasites [17] have failed to prevent ring form development to the mature "adhesive" *P. falciparum* parasite stage. This suggests that the later stage specificity is intrinsic to the drug and does not reflect resistance. Halofantrine was almost as effective as the artemisinin derivatives (particularly against rosetting), yet a parenteral formulation is not generally available, and the oral bioavailability is low and erratic, so it cannot be used for the treatment of more severe malaria.

In clinical trials, the artemisinin derivatives have given consistently faster clinical and parasitologic treatment responses compared with other antimalarial drugs. In general, artesunate has produced more rapid clinical and parasitologic responses than artemether and may be intrinsically superior [21]. The rapid inhibitory effects of these drugs is associated with increased clearance of ring form–infected erythrocytes. Thus, parasites are prevented from developing pathologic characteristics, and significant numbers are removed from the circulation before these characteristics develop [11]. We recently showed that oral artesunate offers a considerable advantage in speed of recovery compared with an intravenous quinine loading dose in children with high parasitemias and no evidence of vital organ dysfunction [22]. Such patients are at risk of developing complications if their heavy parasite biomass continues to mature and multiply. However, although the artemisinin derivatives have many favorable pharmacodynamic characteristics, it is not known whether they can reduce the mortality of severe malaria. Further studies to determine the efficacy and consequences of preventing cytoadherence and rosetting in severe and complicated malaria will help define the role of these drugs in malaria treatment.

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